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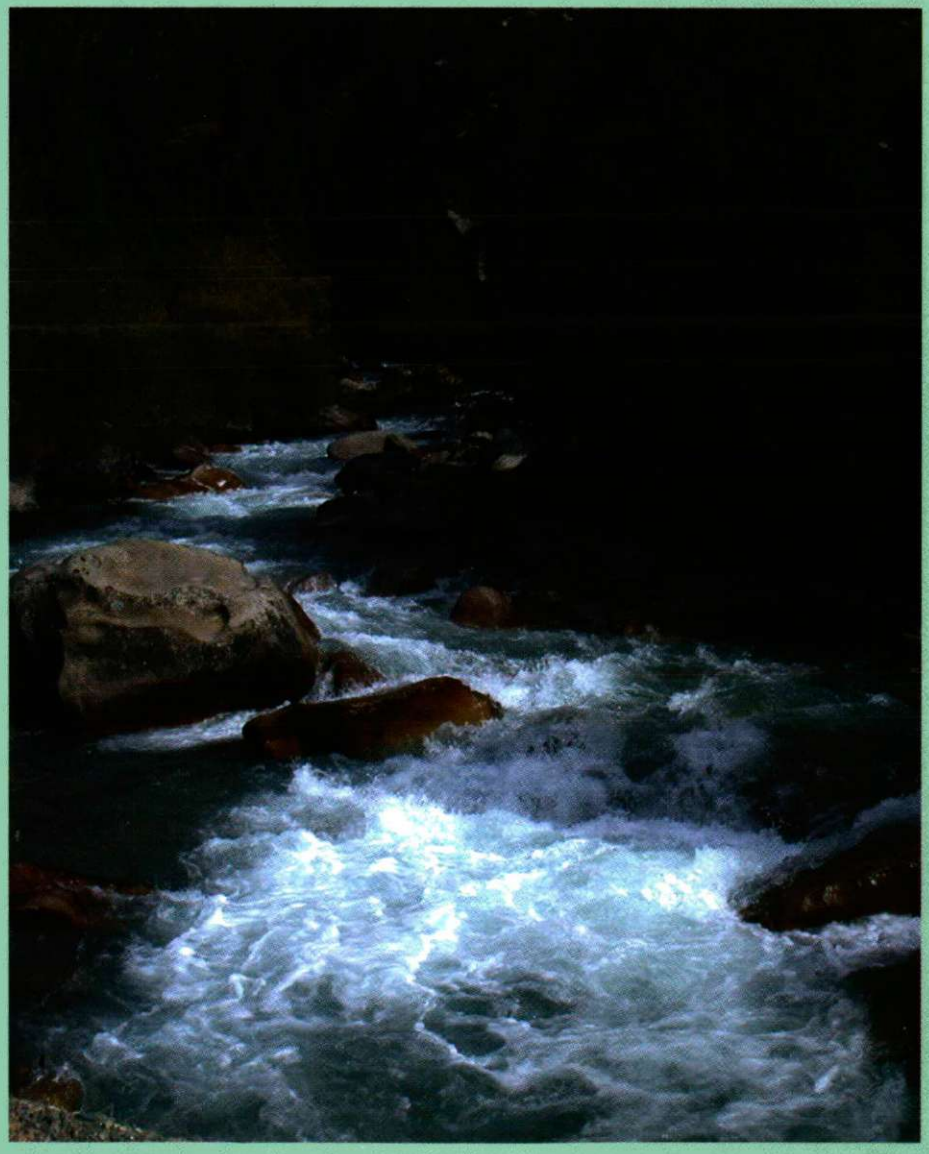

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Acta Biologica Szegediensis

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On the cover: A clean stream in the Tien Shan mountain region, Kyrgyzstan, see in this issue Frimmel (2006) Acta Biol Szeged 50(3-4). Photo: Dr. László Erdei

EDITORIAL

This issue of *Acta Biologica Szegediensis* announces the appointment of two Editors-in-Chief and a new Associate Editor, dr. Maria Szücs. The rapid advances in the biological sciences have necessitated a broadening of the expertise of the editorial body of the journal. Instead of the previous four issues, *Acta Biologica Szegediensis* will in the future be published in two issues yearly. The journal will continue to devote particular attention to the growth areas of modern biology and will publish original research articles concerning, but not restricted to, the fields of anatomy, embryology and histology, anthropology, biochemistry, biophysics, biotechnology, botany and plant physiology, molecular plant biology, all areas of clinical sciences, conservation biology, ecology, genetics, microbiology, molecular biology, neurosciences, paleontology, pharmacology, physiology and pathophysiology, and zoology. We shall likewise continue to publish review articles and, occasionally, Supplement issues with conference material addressing new developments in a particular field of biology. We are excited about the possibilities for *Acta Biologica Szegediensis*. However, in order to improve the quality and standing of the journal, there will be a constant need for active collaboration from our authors who submit excellent work, from our dedicated reviewers, and from our Associate Editors.

László Erdei and Karoly Gulya
Editors-in-Chief

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ARTICLE

Mad cow disease

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ABSTRACT The correct name for Mad Cow Disease is Bovine Spongiform Encephalopathy (BSE) and I will use this abbreviation throughout the text. BSE was first detected in England in 1985. Since then millions of cattle have been slaughtered either because they were infected or for precautionary measures. Payment for compensation to the farmers has cost the UK Government some Euro 6 billion. Although the UK has been the main site for BSE other countries have had BSE and all countries are forewarned. It seems certain that BSE has been transmitted to humans which has emphasised the necessity to protect the public from further infections. The many measures taken will be described. Basic research continues in an attempt to understand the science behind the advent of BSE but there remain many puzzling aspects. Some success has been achieved in identifying infected animals before the clinical symptoms appear. Naturally many professional people have been criticised for the spread of BSE and interesting lessons are being learnt from the links between politicians and scientists. There is little doubt that the standing of scientists in the public eye has been detrimentally affected by BSE.
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KEY WORDS

BSE
Creutzfeld-Jakob disease
prion
scrapie

It is many years since I first lectured on this subject which now has a mammoth bibliography and indeed publications reporting new developments appear daily. This, then, is merely an attempt to provide the background on which opinions about food safety should be based. After a brief introduction I will describe the epidemiology of BSE, then summarize the basic science and the gaps in our knowledge concerning the infectious agent; I will then summarize the precautions that have been taken and end with the impact of BSE on the reputation of the scientists and their interaction with the politicians. I should mention my credentials, or lack of them, for I have retired from experimental work for many years. My research interests were on the biosynthesis of animal proteins so I have for long been interested in the disease of sheep known as "scrapie" and this has led to an interest in "BSE". We have many experts on the subject at University College London and I have endeavoured to keep abreast of what has become a major field of international research. I will not provide an extensive list of references but for background information the recent book edited by Prusiner (2004) should be referred to.

Epidemiology

The situation concerning the occurrence of the transmissible spongiform encephalopathies (TSE) in the early 1980's is summarised in Table 1.

Scrapie in sheep is characterised by an irritation of the skin caused by damage to the neuronal cells, which causes the animals to rub against a fence or wall in the later stages of the clinical condition, hence its name. While there are sporadic outbreaks of scrapie in many countries, including Europe and some Americas, it is no longer present in Australia or the USA, some breeds being very resistant. Scrapie could be transmitted to other sheep by intracranial injection of infected brains but more significant was the transmission to other species such as goats. After the first passage to a goat there is a long incubation period. This so-called "species barrier", whereby the clinical symptoms of the disease take longer to emerge when the recipient is of a different species from that of the donor, is an important characteristic of the disease. The length of the incubation period is related to the evolutionary gap between donor and recipient. In 1961 it was shown that the infection could be transmitted to mice with a much shorter incubation period and more certain outcome and they, therefore, became a favourite test animal. The Syrian Golden Hamster has also proved to be a useful experimental animal particularly in the hands of Prusiner in San Francisco.

Table 1. The occurrence of transmissable dementias.

Species	Name of dementia
SHEEP	Scrapie
HUMANS	Kuru in New Guinea
	Creutzfeldt-Jakob Disease (CJD)
	Gertsmann-Straussler-Scheinker (GSS)
	Fatal familial insomnia (FFI)
COWS	Bovine Spongiform Encephalopathy (BSE)

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Table 2. The chronology of the BSE Epidemic.

Date	Event
1985 April	First clinical observation
1986 November	Disease identified as BSE
1987 December	Meat and Bone meal (MBM) implicated
1988 July	MBM feed banned
1988 August	All diseased cattle slaughtered
1989 February	Risk to humans "remote", Southwood Committee
1989 November	Offal banned for consumption
1992	BSE epidemic peaks at 36,681 cases in year
1995 November	Deaths of 3 young patients
1996 March	Suspected link between BSE and nvCJD
1996 July	Controls on slaughter of sheep

In humans Zigas and Gajdusek discovered a disease named "Kuru" among the Fore tribe in New Guinea. They showed that this was spread as a result of a cannibalistic feast involving ritual consumption of their dead relatives. The explanation of Kuru was that by chance someone suffering from CJD, which as I will explain is a nervous disease occurring sporadically all over the world, appeared among the Fore people. In view of the work with scrapie, Gajdusek inoculated chimpanzees and other primate species with suspensions of Kuru brains. The chimpanzees succumbed after about 1.5 years. Gajdusek was awarded a Nobel prize in 1976.

Until 1985 there were probably not many biochemists in the UK who were more than vaguely aware that sheep tended to suffer from scrapie. Moreover, since the disease had existed in the UK for some 200 years, and no one had suggested that it could be transmitted to humans, the subject was hardly a prominent one for research. Nevertheless, the British Government did continue to finance research at a modest level in order to unravel the nature of the infective agent, often described as a "slow virus", and similar work was pursued in other countries, especially the USA.

All this changed following the report in 1985 from a farm in Kent that they had a cow that had difficulty in walking and suffering from what is now called "Mad Cow Disease". After a delay of about a year before the full significance of the finding became apparent the situation changed dramatically. The disease became known as "Bovine Spongiform Encephalopathy" in view of the spongy appearance of sections of the brains of the dead animals.

The chrononology of the epidemic of BSE

The number of cases of BSE rose dramatically and investigations to study the cause were set up. The critical dates in the unravelling of the epidemic are given in Table 2.

Suspicion centred on a high protein dietary supplement prepared from meat and bone meal (MBM) from the scrapings and offal of cattle and other animals that were not suitable for feeding to humans. MBM was particularly used for feeding to high milk-yield diary cows and it was a common practice in many countries. The traditional way of preparing MBM was to extract the fat with hot organic solvents to give a protein rich product and tallow. Steam treatment was then applied to recycle the organic solvents. Around 1980 an increase in fuel prices made the organic solvent extraction uneconomic and it was omitted. Because of the rise in the price of soya, MBM became more important as an animal feed.

Report of the Southwood Committee

As a result of the suspicion that BSE was caused by the feeding of MBM a ban on its use as a feed for cattle and sheep was rapidly instituted and in 1988 a working party under Professor Southwood was set up. They confirmed in 1989 the suspicion concerning MBM, and recommended that all affected cattle be slaughtered. At that time it was suspected that the trouble was caused by scrapie from infected sheep being transmitted to cattle via MBM but as I will show this is now questionable. As I have said, scrapie had been known for some 200 years and had never been shown to be transmitted to humans. On this basis the Southwood Committee reported that although transmission of BSE to humans was possible and could not be ruled out the chances of it happening were "remote". The British Government eagerly accepted this advice since otherwise the whole dairy industry was in danger. The politicians assured the public that beef was safe to eat especially since measures had been taken to ban the use of MBM. Soon after, the human consumption of brain, spinal cord and other offal was banned. Because the chance of transmission to humans was regarded as remote, it is clear that some of the recommended precautions were regarded merely as "window dressing" and were not fully implemented.

The number of cases of BSE rose dramatically and it is estimated that about 1 million cattle have been infected in the UK and of these about 750,000 were fed to humans in the period 1985-95. Although the incidence of BSE has been greatest in the UK there have been many cases in Switzerland and Portugal and a few cases in many other countries. In addition to the slaughter of animals showing symptoms there has been a ban on the human consumption of cattle over the age of 30 months since the symptoms of BSE mainly occur in older cattle. Such apparently healthy animals in affected herds have been slaughtered and many millions of cattle will have been slaughtered. The British Government has had to pay compensation to the farmers which has cost more than four billion pounds sterling. During this period some 25 million cattle will have been killed for food so the infected animals represent 3% of the total. The measures taken to control BSE in cattle have in general been successful and it is slowly dying out, but more slowly than originally predicted. The more recent figures are shown in Table 3.

The slow decline, with some cases occurring in cattle born after the complete ban on the feeding of MBM in 1996, is a

Table 3. Recent incidence of BSE in cattle in UK.

Year	Number of reported cases
1997	4309
1998	3178
1999	2254
2000	about 2000
2003 Jan.-May	81
2004 Jan-May	38

puzzle and has been attributed by some people to transmission from mother to calf before clinical symptoms have arisen but tests on 23,600 calves born to BSE-infected cattle have produced no evidence of this transmission.

Transmission of BSE to other species including man

After 1987 cases of BSE appeared in various other species of animals particularly various ungula in the zoos and also a few cases in cats. The incidence in cats caused particular concern for it was the first case of transmission to a meat eating animal rather than a herbivore. This strengthened the concern about the possible transmission to humans.

Creutzfeld-Jakob Disease

Apart from Kuru, three different kinds of CJD are recognised. First, there is "sporadic" which is the most common and affects individuals, usually in later life, for no known reason. Those affected reside worldwide and there has been no evidence of a recent epidemic in the UK. Second, "familial", this is an autosomal dominant disease which is rather rare. A good deal of genetic work has been done to understand this. Third, "iatrogenic" which has been caused by the administration of growth hormone preparations, made from a pool of 3000 cadaver brains in the USA, to children with problems of growth. There has been the emergence of CJD in children as a result of such treatment in several countries. The use of recombinant growth hormone has replaced pituitary extracts.

Later a further kind of CJD was to emerge as indicated in Table 4.

In August 1995 the first young person, then aged 19, died of CJD followed by several others who were aged about 29.

Table 4. Comparisons between sporadic and new variant CJD.

	Sporadic CJD	New variant CJD
Mean age at onset	65 years	26 years
Mean duration of illness	5 months	13 months
Presenting features	Rapidly progressive	Anxiety Depression Dementia
Codon 129 genotype	80% Met/Met	100% Met/Met

The brains of these patients had a pathology which closely resembled that of cattle with BSE and was quite different from those who had died of sporadic CJD. In March 1996 when 10 young people had died of CJD the British Government took the advice of their scientific advisory committee that we were witnessing the emergence of a "new variant" of CJD (nvCJD) where the duration of the illness at 13 months was long compared with sporadic CJD at 5 months. It seemed likely that this had arisen by the transmission of BSE as a result of the consumption of infected beef. The long incubation period of nvCJD, probably of at least 5-l0 years, is based largely on the assumption that the greatest chance of people eating infected beef was between 1980 and 1988 when the ban on MBM was instituted. The best present estimates of the maximum number of cases likely to occur is 136,000 but this would imply an incubation period of 60 years which is unlikely but possible. Fortunately, so far the worst predictions have not been fulfilled since the total number of cases in the UK over the last 9 years is 147 but I will indicate later why this number may rise. Nearly all the cases are in the UK, but there have been 6 in France and 1 each in Italy, Canada, Ireland and the USA. Nearly all of the infected people had been in the UK between 1980 and 1996.

The possible origin of BSE

I have indicated that initially the cause of BSE was thought to be the transfer of scrapie to cattle. However, scrapie cannot be transferred to cattle by feeding BSE but BSE can be transferred to sheep by cranial injection. Unlike ungulates and feline species the symptoms of BSE in sheep are similar to scrapie so it has been suggested that perhaps BSE has been misdiagnosed for scrapie (Kao et al. 2002). However, there has been no marked increase in the number of cases of scrapie since 1980 and there is no incidence of the infection of wild sheep by BSE. BSE in cattle and nvCJD in humans bear the hallmarks of BSE rather than scrapie. The symptoms of mice infected with BSE and scrapie can be differentiated so such experiments are in hand but conclusions cannot yet be reached for there is a long incubation time. Thus it now seems more likely that BSE arose, probably on more than one occasion, as a result of an unusual metabolic event in a cow which led to the formation of a biologically active prion. This did not involve a mutation in the suspected infective agent but it may have been a mutation in another protein that influenced the formation of the agent. It has been conjectured that before the method of preparation of MBM was changed the infective agent would have been destroyed before being fed back to cattle but this view is controversial. It seems certain that the feeding of MBM was to blame but the reason is still not clear.

The nature of the infective agent

Following the discovery in 1961 that the scrapie agent could

Table 5. Polymorphism of prion protein at position 129.

The distribution in Caucasians is:
Met/Met 37%
Met/Val 51%
Val/Val 12%

be transmitted to mice, rapid progress was made in determining the characteristics of the infective agent. The agent was resistant to formalin and seemed to have the size of a small virus, e.g. the picornavirus. However, the results of irradiation indicated that the scrapie agent was much smaller than any known virus and corresponded more closely to plant viroids which only contain RNA but unlike them the agent was resistant to nucleases. These were major findings and to the present day no one has been able to demonstrate that a nucleic acid is associated with the infectivity of the scrapie agent. Another important characteristic of the scrapie agent was its resistance to heat. Thus infectivity was retained even if the brain was boiled but infectivity was destroyed at very high temperatures.

Prusiner in California worked with Syrian Golden Hamster adapted scrapie where the incubation period was shorter than in the mouse and the amount of scrapie agent in clinical brain was at least tenfold greater. He came to the conclusion that the main component of the scrapie agent was a hydrophobic protein which polymerised easily. He coined the name "prion" (proteinaceous infective particle) and subsequently isolated protein from scrapie brain which he claimed was the major prion protein. Thus emerged the so-called "protein only hypothesis" for which Prusiner was in 1997 awarded a Nobel Prize. This was a very unexpected claim that the infectivity could be due merely to a protein for we had always implicated a nucleic acid with infectivity.

Another characteristic of the scrapie agent is that it is partially resistant to breakdown to amino acids by Proteinase K with no loss of infectivity. Proteinase K is a fungal enzyme much favoured by protein chemists. This finding has been linked to the fact that in the transmission of BSE the active agent is not destroyed in the gastrointestinal track and rumen of sheep and cattle and that the agent is resistant to the proteolytic enzymes therein.

The structure of prions

I realise that the audience may have only a limited knowledge of biochemistry so I will speak in rather general terms. Proteins consist of chains of amino acids linked together. There are 20 different amino acids in the chain. The chains are twisted and coiled (tertiary structure) similar to that of the wire in the traditional electric light bulb. The order of the different amino acids in the chains (the primary structure) determines the biological properties of the protein. Prusiner determined the primary structure of his prion, designated PrP^{sc}. A protein with the same primary structure was found to be present in many different tissues of the body, designated PrP^{c}. The function of this protein is unknown but seems to be harmless. The difference between PrP^{c} and PrP^{sc} resides in a difference in the tertiary structure. Experimentally it can be shown that the infective process involves the conversion of PrP^{c} to PrP^{sc}. A crucial experiment in support of the "protein only hypothesis" would be to demonstrate the conversion of PrP^{c} to an infectious protein in the test tube. This has not been possible. Proteins with similar properties to the animal prions have been found in yeast and much effort is being devoted to understand the conversion process using yeast as a model.

I should emphasize that there are many aspects of the basic science and pathology of the TSE's that are as yet not understood (May et al. 2004).

The effect of mutations and polymorphism of prions

A lot of work has been done on the effect of mutations in PrP^{c} on the incidence of CJD. There is no doubt that familial CJD is associated with certain mutations and in sporadic CJD they make some difference to susceptibility. Of more significance is the effect of polymorphisms in PrP^{c} at position 129 of the protein as mentioned before and shown in Table 5.

Since virtually all the cases of nvCJD, so far found except one , are homozygous for Met-129 it suggests that such polymorphisms have an influence on the ease of conversion of PrP^{c} to PrP^{sc}. If this be so then it may be that those with Met/Val will emerge with nvCJD after a longer incubation period.

Diagnosis of BSE and the preclinical identification of PrP^{Sc}

There is now an enormous effort being made internationally to find a reliable method for diagnosing the presence of PrPsc in organs from both cattle, sheep and humans. Since such methods will provide considerable profits to the originator it is not always easy to gain access to the details or the results. It seems highly likely that one or more reliable methods will be demonstrated in the near future. While it is possible to prepare an antibody to prions it has proved very difficult to obtain one that is specific for PrPsc. PrPsc can be differentiated from PrPc by dependance on the resistance of the prion to Proteinase K, so that antibody can be used to detect the residue. The European Union now tests all cattle over 24 months old using a method devised by the Zurich group. The Japanese test all cattle irrespective of age. The latest report I have is that since 1987 1200 French cattle have tested positive. It seems that the methods are useful in detecting animals with the disease shortly before the clinical symptoms appear. The antibodies used in these tests are very expensive. A similar method is used to detect the presence of PrPsc in appendices and tonsils of patients. In the latest report there were 3 positives out of 12,500 specimens tested (Hilton et al. 2004). If this ratio

were repeated across the UK then 3,800 people would carry the infective prion. So far only 3 infected Britons have died in 2004 with 5 still alive.

Precautionary Measures

Although the vast majority of cases of both BSE in cattle and nvCJD in humans have occurred in the UK, cases of BSE infected cattle have appeared in other countries, such as France, Switzerland and Portugal. The USA and Canada hoped that they were free of BSE but there have now been reports of a very few cases in those countries. One worry has been that in many countries farmers who detect that an animal has difficulty in walking, called downers, send them off to the slaughter house so the number of cases may be more than those recorded. The conclusion must be that the situation calls for international collaboration.

Action on the farm and at slaughter houses

There seems little doubt that the original source of infection was MBM and so care must be taken that imports of this feed must be checked. One mistake in the UK was to allow the feed to continue to be fed to chickens and pigs until 1996 long after it had been banned for feeding to cattle. It seems likely that some food was diverted to cattle and no doubt the vessels used were contaminated. Since the symptoms of BSE usually appear only in older cows, there has in the UK, been a ban for 8 years on allowing cattle over the age of 30 months to enter the food chain. Some 700,000 cattle a year are now being slaughtered in the UK as a result of this ban. It is claimed by the farmers that the ban should be lifted now that infected cattle can be detected before the clinical signs are apparent. Others argue that the detection methods are not yet sufficiently proven. Ideally the natural history of every cow should be recorded so that if an animal is detected with BSE it's movement will be known and precautions can be taken. The most likely site of contamination is the spinal cord and so measures must be supervised to ensure the careful removal of this tissue in the slaughter house. Such procedures are more difficult in sheep but to be on the safe side sheeps' brains re excluded from the food chain in the UK and France.

Use of surgical instruments

As mentioned already the infective agent proved resistant to normal methods of sterilization. Charles Weissmann has reported on experiments that he is undertaking to determine whether surgical instruments could account for the transmission of infectivity (Flechsig et al. 2001). He has shown that a stainless steel instrument used in human brain surgery for a patient suffering from CJD can transmit the disease to another patient even though the instrument was sterilized with formaldehyde. Working with mice it seems that a mere 5 min exposure to an infected brain is enough to infect the instrument. Formaldehyde sterilization merely causes the protein to become cross-linked. In spite of this, and the apparent tight binding, the protein can create an infective agent probably by causing the conversion of PrP^{c} to PrP^{sc}. It has been recommended that disposable instruments should be used in brain surgery and for the removal of tonsils. Concern is now being expressed about dental surgery involving the removal of infected roots. While formaldehyde is ineffective, the agent is thought to be inactivated either by heating at 132°C for 4.5 h or by the use of M-NaOH. Dental surgeons are being advised to use 4% hypochlorite. The fear is that there may be a bank of human carriers passing on the infection to others.

Blood transfusion

Although the neuropathologies do not have an immunological pathogenesis there is evidence that the immune system is important for transporting infection from the periphery to the brain (Aguzzi 2001). Thus mature B cells are essential for prion accumulation in the spleen. Infectivity in the spleen is associated with B and T lymphocytes and with the stromal fraction which contains follicular dendritic cells (FDCs). It seems that the infective agent is synthesized in mature FDCs and is transferred to splenic lymphocytes which are in intimate contact with FDCs. These results have given cause for concern about blood banks .Recently PrP^{sc} was found in the spleen of a patient who died from an unrelated cause but had received blood from a donor who later developed nvCJD. In this case the polymorphism at position 129 differed from that of the other patients who died from nvCJD. The content of white cells from blood can be reduced before administration (leucodepletion), but this is difficult and expensive. Blood taken from donors in the U.K. will not in future be pooled. (In the USA people who have lived in the UK for more than 6 months are excluded as blood donors.) In all 15 blood donors in the UK have gone on to develop nvCJD. At least 48 people have received possibly contaminated blood and 17 are still alive. One person appears to have died as a result of blood transfusion. People who have received blood are being eliminated as blood donors.

Blood products

It has been traditional to prepare essential products from pooled blood. Very large quantities of Serum Albumin are used throughout the world. As a result of BSE such products are banned for export from the UK. Fetal calf serum is also used in the preparation of many vaccines and this has also caused concern. Another product, Gelatin, is used extensively in medical preparations and is prepared from cattle bones. Tests have indicated that the prions would be destroyed during preparation but it is also no longer sourced from the UK (Grobben et al. 2004).

Possible therapies

So far all therapies have failed. The Koreans in December 2003 announced the birth of four cloned calves lacking prions. Potentially this represents a way of ensuring that cattle do not have BSE but the expense of producing herds of cattle lacking prions is formidable. Moreover, we do not yet know the biological properties of PrP^c.

Interactions between journalists, politicians and scientists

I have explained some of the many scientific surprises that have arisen as a result of the BSE crisis that has cost the UK government more than 4 billion pounds sterling alone in compensation to farmers and caused much disquiet in agriculture. The public were surprised that meat in the form of MBM should have been fed to herbivorous cattle and even more surprised that sheep with scrapie entered the food chain. It seems that not even the farmers knew the origin of MBM, merely that it was a high protein supplement fed to diary cows. It is good if we have learnt to be more enquiring. The scientists have also been criticised for misleading the public in saying that the transfer of BSE to humans was only a remote possibility. There is no doubt that the reputation of scientists has been badly affected by the BSE saga and we know that the story is not at an end. The experience with BSE has increased the resistance of the public to biotechnology in general.

Journalists rightly reflect the concerns of the public and seek the views of the scientists. They understandably relish straight answers. If a scientist is asked "is it safe to eat beef?" the scientist is unlikely to give a straight answer, Yes or No, but will wish to introduce qualifications. The matter of risk factors may well be referred to. This is an area little understood by the public for the risk they are willing to take depends on the pleasure they derive from the activity. The scientists may mention that while it is very regrettable that some 150 people have died from infection with the BSE agent the numbers are very small compared to the once threatened disaster and the number of people killed on the road. There must be plenty of people who have declined to eat beef but continue to smoke cigarettes.

Lord Waldegrave, a former Cabinet Minister in the British Government, has recently written an article "When scientists advise politicians" and several times refers to the problem of BSE. He correctly emphasises the provisional nature of scientific conclusions and being aware that scientists change their minds, as I have indicated in this article. It is important too, he writes, that Ministers should listen to dissident scientists who, although they may be bad scientists, happen to have got something right. It is not surprising that politicians, having consulted the scientists, have difficulty in conveying the answers to the public, especially when the outlook for a major industry may be affected by their words.

I suspect that the public thinks that the scientists understand the basic science underlying the BSE crisis and that their hedged answers to questions are a cover up. I hope that I have shown that this is not so and that the scientists involved in research on BSE often are dealing with the world of the unknown. For such reasons alone I hesitate to apportion blame among those involved, both scientists and politicians. For more details about this matter the Phillips Report (www.bseinquiry.gov.uk/report) should be consulted

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†Obituary

Peter N Campbell (1921-2005)

Professor Peter N. Campbell passed away on February 7 2005. He was Chairman of the Department of Biochemistry at the University of Leeds, 1967–1975, and Director of the Courtauld Institute of Biochemistry, Middlesex Hospital Medical School, 1976–1987. Since retiring he has been located at University College London. There he was concerned with the 'Scientific Apparatus Recycling Scheme' for FEBS and with the UNESCO 'Molecular Cell Biology Network' in support of biochemists in Africa. He also worked for the Association of Researchers in Medicine & Science. He played major roles in the Federation of European Biochemical Societies (FEBS). Peter Campbell is the author of a large number of scientific papers. He has been Editor of the Essays in Biochemistry series (1965-1985) and Editor-in-Chief, Biotechnology and

Applied Biochemistry from 1981 to 1995. He also edited or authored a number of books, including the Oxford Dictionary of Biochemistry and Molecular Biology.

Peter Campbell travelled intensively in the name of several organizations. His activities as a scientific voyager are summarised in his book "A Biochemical Foreign Correspondent- Stories and Impressions from Around the World" which appeared in 2003. We, the participants in the NATO Advanced Research Workshop on "Food Safety and Security" held between 13-15 September, 2004, at Lake Issykkul, Kyrgyzstan, have had the luck to enjoy his brilliant personality. We all deeply regret the loss of a very valid scientist and teacher.

Extracted from the obituary written by Angelo Azzi, Secretary General of SFRR International and Chairman IUBMB Publication Committee and modified by László Erdei.

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ARTICLE

Drinking water, the most essential and not replaceable foodstuff – legal situation and assessment

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ABSTRACT The availability of water on the globe shows great regional differences. Due to the vital importance of water, quality criteria have been developed for its human consumption. In Europe the Water Directive gives the frame for national legislation concerning public water supply. Lists with parameters and highest acceptable concentrations are given as well as control and management principles to guarantee the health supporting function of water. The occurrence of different pollutants like pathogenic microorganisms, pesticides, pharmaceuticals, heavy metals and persistent organic chemicals clearly shows that water has to be treated before usage. Basis for this is a positive list of compounds including impurities and allowed amounts of added substances as well as remaining concentrations after separation. One of the highest objectives is to come to a sustainable water management. Green production, ecological product design, and consumer information are promising aspects to reach that goal.
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KEY WORDS

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Water is the basis for life. It is the unique, most essential food stuff which cannot be substituted. There are great regional differences in the availability of water on the globe. It is obvious that in addition to the climatic situation the amount of available water is of highest importance for the standard of living of the population. The Food and Agricultural Organization (FAO) defines the necessary availability of water in countries to be 1000 m^3 per capita and year. Countries below that value suffer from water shortage. In many parts of Europe, the available amount of water is around 2000 m^3 per capita and year, whereas in arid zones only a few 100 m^3 are typical (UNESCO-WWAP 2003).

These values seem to be rather high in light of the two liters per day of drinking water which are necessary for the survival of a human being. However, it has to be kept in mind that for hygienic purposes and a well accepted standard of living the water demand is much higher and commonly between 100 and 200 liters per person per day in industrialized countries (BGW 1995). In addition, the virtual amount of water necessary for the production of goods of our daily life has to be taken into account, e. g. for 1 kg of bread on our table, the equivalent amount of 1 m^3 water is needed for growing the corn, harvesting, baking and distributing the product (Zehnder et al. 2003). Due to the importance of water for drinking purposes and irrigation, the quality criteria for human consumption are of vital importance and are subject to ordinances and technical guidelines.

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Raw water situation

Wherever possible, ground water is used as raw water for human consumption. The situation is reflected in the amounts of ground water used for public water supply in the European member states (Table 1).

The reason for the preferential usage of ground water is the high quality of water in aquifers which are well protected by the overlaying soil in which organic pollutants get trapped by adsorption and are biodegraded by microorganisms (Matthеß et al. 1992). In addition, the porous material of the aquifer has an efficient filter function for nonliving matter as well as organisms including pathogenic bacteria and viruses (Ward et al. 1985). As a consequence, favorable natural conditions and a circumspect management of the ground leads to the situation where ground water quality is good enough to allow a direct water consumption without any pre-treatment. This

Table 1. Relative amounts of ground water used for public water supply in Europe.

Country	Fraction of groundwater
Austria	99 %
Denmark	99 %
France	61 %
Germany	72 %
Italy	85 %
Netherlands	69 %
Norway	13 %
Spain	21 %
Sweden	24 %

Table 2. Contents of the annexes of the drinking water ordinance.

Annex No.	Content	Number of parameters
1.1	Microbiology	3
1.2	Microbiology	6
2.1	Chemistry (no change in distribution system)	14
2.2	Chemistry (change in distribution system expected)	12
3	Indicators	20
4	Scope and frequency of examinations	
5.1	Specific analytical methods	
5.2	Specific quality criteria for analytical methods	
6	Treatment Chemicals for Special Cases	3

has been the case even in general major cities of Germany like Berlin, Hamburg and München (Frimmel and Gordalla 1996). Well protected ground water with high hygienic, chemical and physical quality therefore has become a reference material for safe and good drinking water.

In regions where ground water of high quality is not available, cleaned surface water is used for consumption. In highly populated areas, drinking water is often also provided by means of pipelines from places were water resources are sufficiently available.

Surface waters normally are more prone to pollutants than ground water. A careful management of the aquatic system concerned including the watershed, and the availability of several ten meters of depth as zone of self purification makes lakes and reservoirs more favorable than rivers as resources for human consumption. In all cases, however, treatment processes have to be applied to guarantee the hygienic and other quality criteria for drinking water (Frimmel 1999). These quality criteria are given in official directives, rules and laws.

Legal situation

The global importance of drinking water quality is reflected in the "Guidelines for Drinking Water Quality" of the World Health Organization (WHO 1993). The parameter values given are an orientation for all nations. In the United States of America drinking water quality issues are addressed in several rules (e. g. lead and copper rule, disinfection byproduct rule etc.; Roberson 2003). In Europe, in 1992 a Directive of the council was issued concerning the "Quality of Water intended for human consumption" (Council Directive 98/83/EC). This directive had to be transferred into national law by the EU member states and was enforced on January 1, 2003 (Grohmann 2003). The following principles were applied:

The framework of the national law had to closely follow the EU Directive.

Wherever possible and in accordance with the Directive, national law should be used to maintain the high level of water quality given in a member state, *i. e.* better: yes, worse: no.

The responsibilities for meeting the quality criteria and for actions in case of transgression have to be exactly defined.

The overall aim of the law is to guarantee the availability of safe water for public consumption without any concerns. This means that the water has to be "clean", free of any unnecessary and unwanted pollution of microbial, chemical or other kind which can harm the health of the population.

Parameter values

The backbone of the resulting law consists of several lists of parameters and values to guarantee the protection of human health. In addition, rules for practical monitoring and remediation actions, information of consumers and reporting to the EU-Commission are given (Table 2).

The microbial parameters are given in detail in Table 3.

This is a fairly limited list, and it is questionable whether these parameters reflect all the important aspects of modern

Table 3. Microbial parameters of the Directive, their parametric values and specified methods for their determination. CM: check monitoring required; AM: audit monitoring.

Parameter	Monitoring	Protocol	Parametric value	
			Water from distribution network	water offered in bottles or containers
Escherichia coli (E. coli)	CM	ISO 9308-1	0/100 mL	0/250 mL
Enterococci	AM	ISO 7899-2	0/100 mL	0/250 mL
Pseudomonas aeruginosa	CM	prEN ISO 12780		0/250 mL
Colony count 22 °C	CM	prEN ISO 6222		100/mL
Colony count 37 °C	CM	prEN ISO 6222		20/mL
Clostridium perfringens [a)b)]	CM	Given in the Directive	0/100 mL	
Coliforms [b)]	CM	ISO 9308-1	0/100 mL	0/250 mL

[a)] only to be measured as an indicator parameter for waters originating from or influenced by surface water
[b)] indicator parameter

hygienic assessments. It has become clear that E. coli, though not necessarily pathogenic, is a very useful indicator for the recent contact of water with excrements of humans or warm-blooded animals. This bears the risk of infection and cannot be accepted according to the precautionary principle. Clostridium perfringens may indicate the non-vegetative forms of parasitic protozoa, e. g. cryptosporidium or giardia. As can be seen from Table 3, more stringent parametric values have to be applied for water distributed in bottles or containers, e. g. on ships.

The chemical parameters and their values given in Table 4 are set mostly beyond acute toxicological relevance to human beings. According to the precautionary principle, a lifelong (70 years) daily intake of drinking water shall not be injurious to the consumer's health. Some of the substances regulated neither are constituents of the raw water nor environmental water contaminants, but stem from materials used for treatment or distribution of water (Grohmann 2003). This applies also to the disinfection by products (DBP) bromate and trihalomethanes, to PAH and especially to Benzo(a)pyrene which may be set free from coal-tar lined water mains. The parameter values of epichlorohydrin, acrylamide or vinylchloride have to be seen as rest monomers based on the maximum deliberated amount from the respective polymer application. Nitrite is largely formed in the distribution system when chloramination is used as disinfection method. Its limit is considered in combination with nitrate. Furthermore, the limit at the consumer's tap is less stringent than the one at the water works exit.

The so-called indicator parameters (Table 5) are a seemingly unsystematic suite of properties. They mainly refer to aspects of aesthetics, treatment or corrosiveness of the water. Most of them are of minor health relevance, but non-compliance indicates that something with the water handling – watershed, catchment, treatment, distribution – is in disorder.

Compliance of the distributed water with the microbiological, chemical and indicative parametric values of the Directive is assumed to grant that drinking water is "wholesome and clean". The standards refer to substances likely to be present in raw water. Should water intended for human

Table 4. Chemical parameters, their parametric values and the minimum requirements for accuracy of applied analytical methods as specified in the Directive. Percentages refer to the parametric values. LOD: limit of detection, % of parametric value; CM: check monitoring required; AM: audit monitoring.

Parameter	Monitoring	Parametric value	Accuracy	Precision	LOD
Acrylamide	AM [0)]	0.10 µg/L	-[a)]	-[a)]	-[a)]
Antimony	AM [+)]	5.0 µg/L	25 %	25 %	25 %
Arsenic	AM [+)]	10 µg(L	10 %	10 %	10 %
Benzene	AM [0)]	1.0 µg/L	25 %	25 %	25 %
Benzo(a)pyrene	AM [+)]	0.01 µg/L	25 %	25 %	25 %
Boron	AM [0)]	1.0 mg/L	10 %	10 %	10 %
Bromate	AM [0)]	10 µg/L	25 %	25 %	25 %
Cadmium	AM [+)]	5.0 µg/L	10 %	10 %	10 %
Chromium	AM [0)]	50 µg/L	10 %	10 %	10 %
Copper	AM [+)]	2.0 mg/L	10 %	10 %	10 %
Cyanide	AM [0)]	50 µg/L	10 %	10 %	10 %
12-Dichloroethane	AM [0)]	3.0 µg/L	25 %	25 %	10 %
Epichlorohydrin	AM [+)]	0.10 µg/L	- [a)]	- [a)]	- [a)]
Fluoride	AM [+)]	1.5 mg/L	10 %	10 %	10 %
Lead	AM [+)]	10 µg/L	10 %	10 %	10 %
Mercury	AM [0)]	1.0 µg/L	20 %	10 %	20 %
Nickel	AM [+)]	20 µg/L	10 %	10 %	10 %
Nitrate	AM [+)]	50 mg/L	10 %	10 %	10 %
Nitrite	CM [c)]	0.5 mg/L	10 %	10 %	10 %
Pestidices – individual	AM [0)]	0.10 µg/L	25 %	25 %	25 %
Pesticides – total	AM [0)]	0.5 µg/L			
Polycyclic aromatic hydrocarbons (PAH)	AM [+)]	0.10 µg/L	25 %	25 %	25 %
Selenium	AM [0)]	10 µg/L	10 %	10 %	10 %
Tetrachlorethene and trichlorethene	AM [0)]	10 µg/L	25 %	25 %	10 %
Trihalomethanes (THM) – total	AM [+)]	100 µg/L [G)]	25 %	25 %	10 %
Vinylchloride	AM +)	0.50 µg/L	- [a)]	- [a)]	- [a)]

[0)] no changes expected after central treatment
[+)] increase expected in distribution system
[a)] to be controlled by product specification
[b)] ρ(nitrate)/50 + ρ(nitrite)/3≤1 mg/L
[c)] CM necessary only when chloramination is used as a disinfectant
[d)] 0.10 mg/L at ex treatment works
[G)] Germany: 50 µg/L

Table 5. Indicator parameters, their parametric values and the minimum requirements for accuracy of applied analytical methods if specified in the Directive. Percentages refer to the parametric values. LOD: limit of detection; CM: check monitoring required; AM: audit monitoring.

Parameter	Monitoring	Parametric value	Accuracy	Precision	LOD
Aluminium	CM [a]	200 µg/L	10 %	10 %	10 %
Ammonium	CM	0.50 mg/L	10 %	10 %	10 %
Chloride	AM	250 mg/L	10 %	10 %	10 %
Clostridium perfringens [b] including spores	CM	0/100 mL	Protocol given in the Directive		
Colour	CM	Acceptable to consumers and no abnormal change	-	-	-
Conductivity	CM	2500 µS/cm at 20 °C	10 %	10 %	10 %
Hydronium ion concentration	CM	6.5≤pH≤9.5	0.2	0.2	
Iron	CM [a]	200 µg/L	10 %	10 %	10 %
Manganese	AM	50 µg/L	10 %	10 %	10 %
Odor	CM	Acceptable to consumers and no abnormal change	-	-	-
Oxidizability	AM	5.0 mg/L O_2	25 %	25 %	10 %
Sulfate	AM	250 mg/L	10 %	10 %	10 %
Sodium	AM	200 mg/L	10 %	10 %	10 %
Taste	CM	Acceptable to consumers and no abnormal change	-	-	-
Colony count 22 °C	CM c)	No abnormal change	prEN ISO 6222		
Coliform bacteria	CM	0/100 mL	ISO 9308-1		
Total organic carbon (TOC) [d]	AM	No abnormal change	-	-	-
Turbidity	CM	Acceptable to consumers and no abnormal change [e]	25 % [f]	25 % [f]	25 % [f]
Tritium	- [g]	100 Bq/L	- [g]	- [g]	- [g]
Total indicative dose	- [g]	0.10 mSv/a	- [g]	- [g]	- [g]

[a] CM only when used as flocculant
[b] only to be measured for waters originating from or influenced by surface water
[c] CM only for waters offered in bottles or containers
[d] need not to be measured for supplies less than 10000 m^3/d
[e] ≤ 1.0 NTU at ex treatment works should be striven for in case of surface water treatment
[f] specifications must be met only for monitoring of treated surface water
[g] frequency and methods are still to be fixed

consumption however be a human health risk because of other infectious, toxic or undesirable ingredients not mentioned in the Directive, it must also not be distributed to the consumer and, if necessary, the Member States must set values for other additional parameters.

Quality assurance of the data

The results of microbiological tests and of chemical and physical determinations depend strongly on the protocol and methods applied. The quantitative data therefore have to be obtained according to standardized methods like CEN or ISO protocols (Gordalla and Frimmel 2003). Examples for microbiological methods are given in Table 3. The minimum requirements of the data quality to be obtained for chemical and indicator parameters are given in the EU-Directive, and they are shown in Tables 4 and 5. The improvements of the methods applied, and the international standardization of the analytical methods belong to the most challenging fields of analytical sciences.

Frequency of assessment

Analytical data have to be representative. This includes the sampling strategy as well as the scope and frequency of the determined parameters. Samples have to be taken according to standardized methods at a point directly after the water treatment process and at different consumer taps, e. g. in households, schools or public buildings. The sampling campaign frequency depends on the size of the supply system (Table 6).

It is interesting to note that the EU Member States cannot be held responsible for the domestic distribution system nor for its maintenance. Nevertheless they have to inform the consumers about the water quality to enable them to chose appropriate pipe material and operation principles with respect to health and corrosion aspects.

Water treatment substances

If the microbiological and chemical parameter values of the Directive are not met by the raw water, technical treatment

is necessary. The Member States have to regulate the use of substances or materials used for preparing water in a way that possible harmful effects on human health are avoided. These substances have to be applied in a manner that they do not remain in the finished water in concentrations higher than necessary for their use. This also implies minimization of DBPs.

In the Ordinances only for special cases, e. g. natural disasters, epidemics or war-like situations, defined substances and allowed concentrations are given (Table 7).

Substances for water treatment under ordinary conditions are regulated in § 11 of the Ordinance which states that chemicals for water treatment and methods for disinfection need to be declared in a list given by the Federal Ministry for Health and published in the Bundesgesundheitsblatt (Official Journal of Federal Healthcare). The list has to contain substance specific information on

- identity of material
- purity standards
- allowed maximum concentration of application
- allowed maximum concentration of remaining rest substances and reaction products after treatment.

In case of chlorine-based disinfection, minimum concentration of free chlorine after treatment is given. The necessary scope of analytical control of the treatment chemicals is also specified, and methods for disinfection and the conditions for their effective application are defined.

The German list of allowed substances gives 34 different aims for treatment processes and 90 substances at the moment. The list is organized and supplemented by the Environmental Protection Agency (Gordalla and Frimmel 2003). The dynamic character of the list (as non-internal part of the EU Directive and national Ordinance) guarantees its topicality. The permission of substances and materials depends on their technical effectiveness, and on the possible impact on health and environment. These aspects are evaluated by an expert group of representatives of the State, the army, the railway company, water supply specialists and authorities. Specific rules exist for substances and materials accepted in other EU Member by States or other international trade partners. The application of non-specifically allowed substances and methods is not permitted. Legal basis for all these regulations are the safety rules for foodstuff and consumer goods which have a good tradition in industrialized nations.

The aims for water treatment in detail are:

- elimination of unwanted substances from raw water
- alteration of the water constituents to guarantee a safe and technically perfect distribution of the water to the consumer. This can include corrosion aspects and hardness.

Inactivation or destruction of pathogenic agents of water

- in the treatment plant (primary disinfection)
- in the distribution pipes (secondary disinfection)
- in storage tanks (secondary disinfection).

Technical basis for these regulations is the broadly accepted technology. This allows a more generous approach than a state of technology based regulation. Of general importance is the so called 10% rule in combination with the minimization principle (MP). This means that the additional chemical load of drinking water caused by treatment chemicals has to be kept as low as possible. It refers to the added chemicals as well as to their reaction products. In any case chemical additions are not allowed to lead to more than a 10% increase of the original parameter value in the water. This implies of course that the original value is less than 90% of the maximum allowed concentration.

If possible, the control of chemical additions should be done continuously to avoid too high doses. This applies mainly to the additives which remain in the water like phosphate, silicates, chlorine, acids or bases and oxygen. The substances like flocculants and activated carbon which get eliminated after the treatment step are assessed as technically unavoidable compounds and have to be eliminated to technically irrelevant levels which show no adverse health, odor and taste effects. Based on the obligatory technical product quality, standard concentrations also can just be calculated using the manufacturer's product speciation, the amount of chemicals added and the volume of water treated. It is the aim of a dynamic positive list of treatment methods and chemicals to serve the minimization principle. Best of course would be if the raw water quality was good enough to make any addition of chemicals unnecessary.

An important part of the Directive also deals with the information policy of the water supply companies and the responsible authorities on the one hand and the EU Council and the consumers on the other hand. Each significant non-compliance has to be announced and assessed, and strategies of tackling the specific problems have to be supplied in due time.

Existing and emerging problems

There exists a number of actual problems in public water supply. Despite of a broad awareness of the importance of the hydrological cycle and the needs of raw water protection for the supply of people with the fundamental foodstuff water, there are numerous examples of severe problems in water quality. Some are addressed here:

The pollution of groundwater by pesticides and nitrate in many regions is caused by intense farming. Pesticides in their manifold chemical structures have a maximum allowed concentration in drinking water of 0.1 µg/L each which is often exceeded. Toxicologically relevant metabolites also have to be taken into account. The parameter value for nitrate in the Directive is 50 mg/L. We have learned that groundwater polluted with persistent chemicals can take decades to recover after the

Table 6. Frequency of sampling and analyses depending on the volume of water supplied from a distribution network.

Volume V of water distributed of produced each day within a supply zone	Check monitoring number of samples per year	Audit monitoring number of samples per year
$V \leq 100\ m^3$	Frequency to be decided by the Member State concerned	Frequency to be decided by the Member State concerned
$100\ m^3 \leq V \leq 1\ 000\ m^3$	4	1
$1\ 000\ m^3 \leq V \leq 10\ 000\ m^3$	4	1 +1 for each 3 300 m^3/d and part thereof of the total volume
$10\ 000\ m^3 \leq V \leq 100\ 000\ m^3$	+3 for each 1 000 m^3/d and part thereof of the total volume	3 +1 for each 10 000 m^3/d and part thereof of the total volume
$> 100\ 000\ m^3$		+ 1 for each 25 000 m^3/d and part thereof of the total volume

pollution source has been eliminated (LfU 2003). This also underlines the importance of the metabolites of the pesticides. This situation asks for a powerful ground water protection and a ground water related enforcement of good farming practice. The dilemma is clear: we all want to have rich crops and enough meat for our nourishment, but we and the next generation also urgently need clean water as basis for our life. Recently, a biodegradable polymer (poly(ε)caprolactone) has been introduced as promising absorbant for pesticides which, mediated by the biodegradation, leads to a denitrification and cometabolic pesticide elimination. In polluted waters this can help to solve both problems at a time.

Another reason for concern lies in highly populated areas and their growing needs for water which is taken from poor aquifers. Taking more water out of an aquifer than the amount which is recharged leads to a lowering of the water table and finally to the use at lower water levels to overcome the shortage. The hydrogeology in the deeper underground offers water with higher salt content, higher hardness and in some regions higher arsenic concentrations (Bissen and Frimmel 2003). All these parameters can exceed the values given in the Directive.

Industrialization with uncontrolled left-overs and dumping areas have led to so called hot spots with high risk potential for the groundwater. Polycyclic aromatic hydrocarbons (PAHs) and other persistent pollutants as carcinogenic and mutagenic compounds are dominant. Benzo(a)pyrene with 10 ng/L is the lowest parameter value of the whole Directive. Amongst the different technical remediation techniques, natural attenuation methods have become most attractive for economic reasons (Förstner and Gerth 2001). However, in these cases thorough analytical control instead of complicated technical treatment facilities has to be installed.

An interesting emerging problem can be seen in the environmental occurrence of pharmaceuticals and their metabolites. Lipid regulators, anti epileptic drugs, contraceptives and even iodinated organic X-ray contrast media have found their way via waste water, rivers, lakes and the ground water to the water supply systems (Daughton and Ternes 1999, Drewes et al. 2003). These compounds so far have not been regulated by parameter values. Their subtle effects in water are less in the range of acute toxicity, but more in the field of chronic effects. Identification, quantifycation and assessment of pharmaceuticals in the aquatic environment are a challenge for the years to come.

DBPs are another important topic in water supply. There are only two parameter values, the THMs (with 100 µg/L) and bromate (with 10 µg/L) in the Directive so far. Far more than hundred of compounds have been identified up to now (Richardson 2003). Some of them like MX have turned out to be highly mutagenic (Barrett et al. 2000). A good part of the DBPs formed are still unidentified. In the past, especially the polar ones were difficult to be analyzed. The availability of high performance liquid chromatography (HPLC) coupled with tandem mass spectrometry (MS/MS) has recently

Table 7. Chemicals for treatment in special cases.

No.	Substances	EWG no.	Aim	Allowed concentration mg/L
1	Sodium carbonate Potassium dichloroisocyanurate		Disinfection	40 [1]
2	Sodium carbonate	500		
	Sodium bicarbonate	500		
	Adipinic acid	500		
	Sodium benzoate	335	Aid for tableting	
	Polyoxymethylenpolyglycol waxes	E 211		
	Sodium chloride Tartaric acid	E 334		
3	Sodium, Calcium, Magnesium hypochlorite	925	Oxidation Disinfection	200 [2][3]

[1] minimum amount: 33 mg/L
[2] as active chlorine
[3] minimum amount: 100 mg/L

opened the door to the analysis of the world of hydrophilic DBPs (Zwiener and Frimmel 2003).

The assessment of the biological effects of the suite of DBPs is still poor and has many open questions. The Directive addresses this situation with the recommendation to enlarge the chemical and microbiological analytical methods if necessary. The dilemma is again obvious: there is a choice of high hygienic safety which is achieved by a high dose of disinfectants and consequently a high amount of toxic and mutagenic DBPs or a low risk coming from the toxicity of DBPs which are minimized by low doses of disinfectants that have only a limited potential for the inactivation of microorganisms.

Finally, it is an urgent issue whether the classical way of assessing the hygienic quality of raw water and drinking water is efficient enough for a reliable health protection of the population. Direct information on parasites and viruses are missing in the Directive. In addition, the time span between sampling and availability of the final results which normally lies in the range of several days is too large to prevent epidemic outbreaks. Modern molecular biological testing and cell tests are promising to add precious information on microbiological pollution in a much shorter time (Grummt et al. 2004). Tailor-made bioassays can also help to bridge the gap between chemical analysis and biological effects which should be a major field for research and development in water hygiene.

Sustainable water shed management

The global water crisis seems to be evident. More and more people have to rely on the limited availability of water, the most essential resource for life. This asks for a convincing strategy to face the future. Sustainable water shed management based on ecological, economical and social aspects can show us the way. All parts of the hydrological cycle have to be seen in this sensitive magic triangle, and wise decisions on the system management have to be made to guarantee a safe water supply also for the next generations. Production-integrated environmental protection, green product design and consumption-integrated environmental protection as well as waste and waste water handling with minimized pollution of aquatic systems must be the key stones of our economic system. Precautionary principles and minimization principles for chemical addition in water treatment processes are essential. Biological, chemical and technical methods based on sustainability are promising to reach a water consumption orientated on natural processes as they are given in the hydrological cycle.

The EU has given a Directive for a framework for community action in the field of water policy (Council of the European Union 2001). It contains the ideas of a definition of safe drinking water which is orientated on a properly managed ground water without any major environmental pollutants. In short: it should be appetizing and it has to be colorless, clear and cool without any adverse odor, taste, or a significant amount of germs (DIN 2000 2000). Consequently, a concerted effort on a sustainable water management has to be undertaken by mankind to support its further existence.

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ARTICLE

Optical chemical sensors and biosensors for food safety and security applications

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ABSTRACT Over the past two decades or so, the incorporation of optical techniques in the development of chemical sensors and biosensors have been investigated resulting in novel and very interesting devices with great promise for many areas of applications. These are truly integrated and interdisciplinary systems that invoke expertise from the fields of chemistry, biochemistry, physics and electronics. Optical chemical sensors and biosensors utilise immobilised reagents and novel materials that can result in a variety of optode designs that are suitable for a variety of measurements in environmental, biomedical, industrial and process control areas.
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KEY WORDS

biosensor
chemical sensor
food safety
optode

Chemical sensors and biosensors are really sensing devices that utilise the principles of optical molecular spectroscopy in conjunction with chemical systems for use in a variety of applications. The term '**optode**' is sometimes used, as in this paper, which represents the combination of **opt**ical measurements with configurations and performance that are similar to electr**ode**. Optodes represent a group of integrated analytical systems, where chemical and biochemical measurements are performed based on the interaction of light with the chemical/biochemical media followed by conversion of optical signals into readable electrical signals. In this group of devices, optical fibres are often employed as the medium that conveys optical signal to the measurement system and to the detection system. Chemical and biochemical media contain immobilised reagents that are capable of interaction with the measurand of interest, and thus provide the molecular recognition element generating optical signals. Thus the optical signal returned from the recognition element will be encoded with chemical/bio-chemical information conveyed by optical fibres to suitable detectors.

The incorporation of optical fibres in optodes imparts a number of advantages in measurements such as miniature device application, geometrical flexibility and ruggedness, small sample volumes, remote signal detection, multisensing capabilities and low cost. In addition, the use of immobilised reagents enables the development of specific and sensitive optodes in several fields of applications. A few disadvantages such as ambient light interference, limited dynamic range, long response times and reagent stability, may exist with optodes. These can be overcome by proper sample preparation and engineering appropriate instrumentation. Optodes have been developed and employed for chemical and biochemical analytes in environmental monitoring, chemical industrial process control, biotechnology (e.g. food science) and biomedicine, and many more are possible. The development of new materials and optode designs is continuously being pursued broadening the scope of applications of optodes.

This paper presents a brief review of the state-of-the-art of this optode technology, and covers briefly the principles and applications of these devices in a number of areas including food safety and security, with an insight to its future trends.

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Principles of transduction

The basic concept of a chemical and bio optode is simple and can be represented as in Figure 1. The molecular/ionic recognition is carried out by the transducer containing immobilised reagents and the measurement involves optical signals that are conveyed by optical fibres. In fact light is launched into the optical fibre from a suitable light source and directed to the measurand region and the returned light is collected by the same or another optical fibre to be measured by the detector. This optical signal may be absorption, emission, transmission, reflection or scattering of light by the transducer in the optode. Various model equations that relate the signal measured to concentration of optical species by the above mentioned techniques of interaction of light have been used (Narayanaswamy and Sevilla 1988). In addition, evanescent waves have been used in optodes, which refers to the light that penetrates into the surrounding medium from optical fibres; have been employed in optode development (Harmer and Narayanaswamy1988, Narayanaswamy 1993a). Such phenomenon is extensively utilised in sensors based on Surface Plasmon Resonance (SPR) of analyte specific coatings

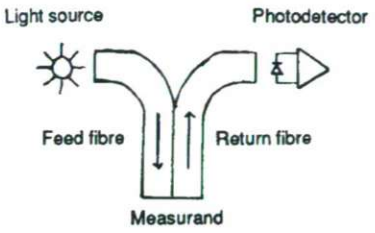

Figure 1. Schematic of an optode (from Narayanaswamy and Sevilla 1988).

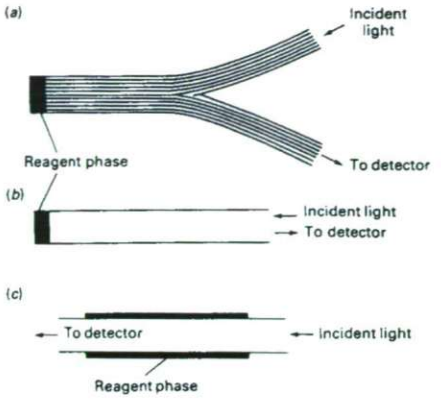

Figure 2. Common optode designs (from Narayanaswamy 1993a).

on waveguides (Harmer and Narayanaswamy 1988).

A variety of sensing mechanisms have been adapted in optodes. These include reversible binding of analyte to a reagent/sensor surface, irreversible chemical interactions between analyte and reagent, specific binding of analytes (e.g. biomolecules) and direct spectroscopic examination of analyte. The sensor chemistry may utilise a single or a mixture of such different mechanisms to produce an overall optical response that can be measured and correlated to analyte concentrations.

Immobilisation of reagents

Immobilisation of reagents for optodes is a key operation that determines a number of their characteristics such as response times, lifetimes and also robustness. They are commonly used in solid state to provide ease of interfacing to optical fibres and this allows simple measurements to be carried out with facilitates the reuse of reagents, if needed.

Physical and chemical methods have been used for the immobilisation of reagents in optodes. Physical immobilisation is achieved through adsorption, entrapment, encapsulation or electrostatic attraction between the reagent(s) and polymeric solid supports(s). In adsorption, the reagent molecules are held on the surface of the support by physical forces such as hydrogen bonding or hydrophobic interactions. In entrapment/encapsulation, the reagents molecules are confined in the lattice structure of the support, while electrostatic attraction involves interactions between ionic groups present in the reagents and in the support material. Some recent work incorporate reagents imprinted on specially designed polymers and this field is of growing interest (Al-Kindy et al. 2000). These physical immobilisation procedures are simple and involve mild reaction conditions. As a result, the binding strength of the reagent molecules on the support will be weak and result in problems of reagent being leached from the support surface. Chemical methods involve the formation of covalent bonds between the reagent and the polymeric support via reactive functional groups such as $-NH_2$, –OH, –CHO, $-SO_3H$, –Cl, –COCl, etc. Reactive polymeric supports that have been used in optodes range from inorganic materials (e.g. glass, sol gel, etc.) to organic polymers (e.g. XAD polymers, nylon, cellulose, silicone, etc.). Chemical immobilisation provides stable reagent matrices for optodes. In addition, polymeric membranes may constitute part of the optode design which can provide analyte selectivity to be achieved and thus, improve the optode performance characteristics.

The reagent immobilised materials can then be fabricated into several configurations such as thin films, solids in columns, gels, etc., to be interfaced with optical fibres.

Optode designs

The integral part of many optode designs incorporates optical fibres, which are either optical fibre bundles or single optical fibres (Figure 2). Fibre bundles are bifurcated so that separate fibres can transmit incident and detected radiation (Fig. 2a), while with single fibres, the transducing element can be interfaced at the end of the fibre (Fig. 2b) or applied as a coating on the fibre surface as a cladding material (Fig. 2c). The reagent cladding-based optode design utilises the evanescent waves of optical radiation passing through the optical fibre. Many other designs have been studied for optodes depending on the nature of their application (Wolfbeis 1991).

Instrumentation

The instrumentation associated with optodes is similar to those of common spectrophotometers, and include components such as a light source, a wavelength selector, a photo detector and a display of output (Narayanaswamy and Sevilla 1988, Narayanaswamy 1993a). In addition, optical components such a lens, optical couplers and connectors are used for coupling of light into optical fibres. Commercial portable instrument systems have been developed incorporation solid-state opto-electronic components (Ocean Optics Inc). A review has been published in the literature that deals specifically with instrumentation for use with optodes (Taib and Narayanaswamy 1995). As far as optical fibres are concerned,

depending on the fibre material, different range of wavelengths of light can be transmitted through the fibre medium. For example, while glass and plastic optical fibres are useful in the visible and near infra-red region of the electromagnetic spectrum, quartz optical fibres can be used to transmit ultraviolet, visible and near infrared wavelengths of light.

Applications

A number of optodes have been developed for monitoring dissolved and gaseous chemical and biochemical analytes in a variety of fields of applications. For example, pH optodes have been fabricated with different transduction systems and optical principles, and all of these involve the use of pH indicators (acid-base type) that have been immobilised using physical and chemical procedures (Swindlehurst and Narayanaswamy 2004). Sensors for CO_2 are based on changes in pH of the environment of the sensing reagent. This has been the basis of development of a CO_2 sensor employing dual luminophore referencing for application in food technology, by measurements of the analyte in Modified Atmosphere Packaging applications (von Bültzingslöwen et al. 2002), which has major advantage in food safety.

Oxygen optodes for biomedical and process control applications have been developed based on the quenching of fluorescence of an immobilised luminescent indicator, yielding good precision. The signals measured are correlated to O_2 concentrations using the Stern-Volmer equation. Measurement of luminescence lifetimes has been the basis of measurements in an oxygen sensor that has been used in biotechnology and food industries (Köneke et al. 1999).

Covalently immobilised enzymes have been used in the development of bio-optodes for glucose, urea, etc. The use of competitive binding reactions of glucose and of fluorescein-labelled dextran with Concanavalin A (antigen) has resulted in a reversible optode system for detection of low levels of glucose. A recent review paper has been dedicated to the use of immobilised enzymes in the development of bio-optodes (Kuswandi et al. 2001).

Heavy metal ions (Al^{3+}, Cd^{2+}, Cu^{2+}, Hg^{2+}, Mg^{2+}, Pb^{2+}, Zn^{2+}, etc.) in the environment and food have been determined using optodes that utilise immobilised chelating ligands that nearly specifically interact with metal ions. These optodes require fairly rigorous control of pH of the medium and can result in devices with very low detection limits. Several optodes have been developed for the monitoring of gaseous and vapour chemical species and these systems could be adapted for detection of the analytes in dissolved state (Narayanaswamy 1993b). Here gases include NH_3, CO_2, Cl_2, NO_2, SO_2, H_2, etc., and vapours such as water, volatile organics. The use of novel electrochromic polymers in the development of gas optodes have been investigated with very reasonable detection levels (Kondratowicz et al. 2001). Humidity sensors, that can be used in the manufacture and storage of food products, has been the subject of a recent review (Moreno-Bondi et al. 2004), and many types of optical humidity sensors have been investigated by many researchers.

Recently, research has been concentrated for developing optode systems that are capable of multi-analysis. In these, novel signal processing techniques have been employed to analyse the differences in absorption/reflectance wavelengths and reaction kinetics. For example, humidity and NH_3 have been simultaneously determined with a single optode based on Nafion® immobilised crystal violet reagent (Raimundo Jr and Narayanaswamy 2001). Simultaneous determination of Zn(II), Cd(II) and Hg(II) in water has been studied utilising a single reagent adsorbed on a polymeric material (Raimundo Jr and Narayanaswamy 2003). The use of integrated technology in a multifunctional biochip has been described recently that utilises two different bioreceptors on a single platform for application in medical diagnostics and quantitative detection of pathogen (Vo-Dinh et al. 2003). Toxins in food have been detected using an optical fluoro-immunosensor in an array biosensor configuration that is capable of detecting multiple targets (mycotoxin, bacterial toxins, etc.) have been investigated (Ligler et al. 2003). An extended study involving the use of SPR biosensors for detection of food safety-related analytes including chemical contaminants, foodborne pathogens and toxins, has been recently reviewed (Homola 2004).

Though publications concerning applications of optical chemical sensors and biosensors for food safety and security have been dearth in the literature, several workers have attempted to use the novel sensor technique as described below. An optical sensor to detect diacetyl vapours that is evolved at the onset of spoilage of meats has been investigated (Shiers and Honeybourne 1993). The detection of bacterial contamination and food processing with optical sensors has been described in a short review presented in a conference (Wolfbeis 1995). Likewise, robust and inexpensive integrated optical sensors based on the use of Mach-Zehnder interferometers have been studied for beverage analysis (Luff et al. 1998). A patent describes the detection of bacterial volatiles in food analysis by using gas sensors and spectral footprints with advantages of identification of particular microorganisms (Alocilja et al. 2002). Based on the analysis of vapours emanating from food, several researchers are currently studying the development of optical sensors, and the results of such work would cause a mini explosion of publications in the near future.

Summary

Chemical and bio optodes that have been developed over the past two decades have shown considerable promise and many more are being researched. The optode devices need to be combined with advanced signal processing techniques (pattern recognition, artificial neural network, principle component analysis, etc.) in order to improve their characteristics

and capabilities. Many new materials such as electrochromic compounds and molecularly imprinted polymers can be exploited in the molecular recognition element of the optode. New sensing schemes and the use of integrated technologies are being investigated that can result is novel and very useful devices for food safety and food security applications.

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ARTICLE

Importance of animal husbandry and production management on food safety in livestock production systems

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ABSTRACT Production and management systems do significantly effect animal health and therewith the use of drugs and prophylactic treatments. This may lead to residues and a change for the worse in food safety. The consequent implementation and use of hygiene and management tools and measures is needed, as well as a combination of these measures with effective programmes (e.g. vaccination, procedure with cleaning and disinfection, all in all out etc.) in order to limit the use of drugs in sick animals so they are used only when absolutely necessary. A better education among producers is also an important step towards higher food safety on farm level

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KEY WORDS

food safety
livestock production systems
management systems
genotype
beef cattle
dairy cattle
chickens, pigs

Food safety is a significant concern of consumers. Ironically, there is an inverse relationship between food safety and consumer concern about food safety. As the food supply has become increasingly safe, people become .... increasingly „hysterical" about what „agribusiness" is doing to their health" (Cheeke 2004)

This statement seems to be really true at least for Western European countries, where food safety is of major concern for consumers, but in reality food became more safe over the last decade. For example in Denmark every year 20,000 samples are taken from fattening pigs at commercial slaughter houses and are tested for antibiotics and chemotherapeutics, hormones and growth promotants, pesticides and heavy metals. Over the last decade only 0.01 to 0.05% positive samples were found (Nielsen 2002) containing antibiotics and/or chemo-therapeutics. However consumers are reacting very sensitive to any anticipated episodic events like the BSE (Bovine spongiform encephalopathy) crises has shown. Thus consumption rates are immediately decreasing (Figure 1).

Because food production is a very complex procedure and products from animals bear a potential health risk for consumers, depending on management qualities on farm and production levels, food safety has to start on the farm. These on-farm efforts will greatly influence everything else that must be done during the processing and distribution of food.

The potential risks for consumers are mainly microbes including zoonotic bacteria (e.g. *Salmonella*, *Campylobacter*, *Escherischa coli*, *Brucella*, *Mycobacteria*), residues or contaminants of feed additives (antibiotics, antiparasitics), growth promotants and various chemicals including pesticides, disinfectants, environmental contaminants. Therefore the ways to ensure food safety have to start in principle on production level. These are:

1. the elimination (e.g. *Mycobacteria*, *Brucella*), control and/or reduction of zoonotic bacteria on farm level (e.g. *Listeria*, EHEC, *Clostridia*, *Salmonella*, *Toxoplasma*, *Campylobacter*),

2. the reduction of prophylactic and therapeutic use of drugs by improving management factors and

the reduction of chemical contamination.

If these ways are followed consequently on farm and all other production levels a number of conflicts are arising. For example the elimination or control of bacteria mostly comes along with an increase of the prophylactic use of drugs and/or of disinfectants. This conflict can be seen for example in Denmark, where antibiotics in feed have been banned -since the year 2000, which lead to an increased use of therapeutics on farms (Figure 2).

The animal husbandry and production management influences food safety in livestock production on different levels. Many factors may have a direct or indirect effect on food safety e.g. the used genotype, the type of farm (organic or conventional, low- or high-Input system), the farmers education, buildings (production technique/ procedure/ stocking rate), feed stuffs, health and hygiene management etc.. In this paper some examples are given from various production systems.

Genotype and food safety

Breeds have genetically based differences in the resistance

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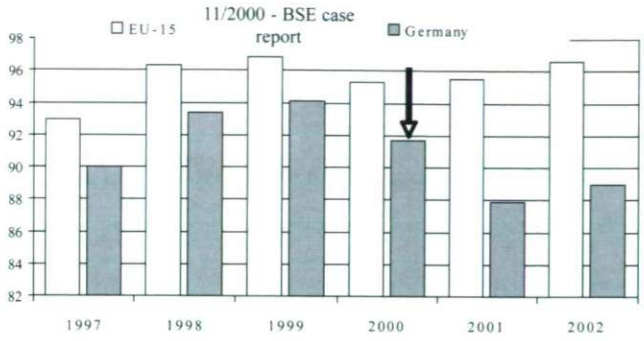

Figure 1. Meat consumption (kg) per capita in the EU-15 and Germany (incl. innards) after first case report of BSE (ZMP, 2003).

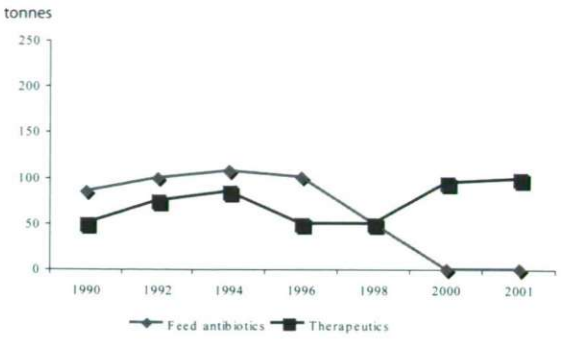

Figure 2. Application of antibiotics in Danish animal production (Danske Slagterier 2002).

against various infections like for example parasites. Pasture management and strategic anthelmintic use are the classical methods to control for example gastrointestinal nematodes. The increase of resistance of internal parasites to anthelmintic treatments and concerns over possible chemical residues, environmental impact and cost of treatments have led to an increasing interest in alternative control methods. The use of more resistant genotypes is one of them. It can lower the total amount of drugs administered and possible chemical residues. Therefore the choice of genotype directly effects the food safety on production level.

Estimated heritabilities for indicator traits of parasite resistance in different species proving the possibility to breed for these traits (Table 1).

Management systems and food safety in beef cattle

Jäger et al. (2005) examined the excretion of faecal stages of different parasites (*Giardia duodenalis*, *Cryptosporidium parvum*, *Eimeria* spp., *Strongyloides papillosus* and S. *strongyles*) and their extensities and intensities in suckler calves of beef cattle herds kept in differenthousing systems. C. *parvum* infections showed for example the highest extensities when animals were kept indoors on deep litter without run-out (Figure 3). Whereas the lowest *Eimeria* extensities and intensities were found in animals kept indoors on slatted floor and animals kept outside. Coccidiosis is one of the most important endoparasitic protozoal infections in calves. It can be a serious clinical problem, causing diarrhoea and reduced growth. Its economic importance lies mainly in the lowered productivity caused by reduced growth rates, delayed age at first service and calving, reduced live performance and total economics. Therefore it has to be treated. The paper clearly showed, that the management system influenced frequencies of subclinical and clinicial diseases and the total amount of drugs needed. Thus an increase of prophylactic and therapeutic use of drugs as consequences on food safety (residues).

Table 1. Heritabilities of indicator traits for parasite resistance in sheep (Haemonchus contortus-resistance) and chickens (Ascaridia galli-resistance) (Gauly et al. 2001, 2002).

Species	Breed/Genotype	Indicator trait	Heritability
Sheep	Merinoland	Faecal egg count	0.17
	Rhön	Faecal egg count	0.12 – 0.35
	Rhön	Haematocrit	0.08
	Rhön	Antibody titer	0.30
Chicken	Lohmann Brown	Faecal egg count	0.10
	Lohmann LSL	Faecal egg count	0.19

Management systems and food safety in dairy cattle

Fossler et al. (2004) studied the connection between the management system and the isolation of Salmonella in dairy cows (n = 20,089) and pre-weaned calves (n = 4,673) on 129 farms. Of the faecal samples taken, 4.9% from cows and 3.8% from calves were *Salmonella* positive. The factors associated with increased odds for *Samonella* shedding were:

no routine feeding of medicated milk replacer,

use of calving pen as a hospital area for sick cows more then once a month,

not storing all purchased concentrate or protein feeds in an enclosed building,

not using monensin in weaned-calf or bred-heifer diets,

Table 2. Frequency of egg contamination with bacteria (E. coli, Proteus and others) in relation to management system (Matthes 1983).

	Free range	Floor system	Cage
Shell surface	53.0 %	28.1 %	11.3 %
Inside shell (bacterial penetration)	5.0 %	2.5 %	0.0 %
Egg yolk	3.1 %	0.6 %	0.0 %

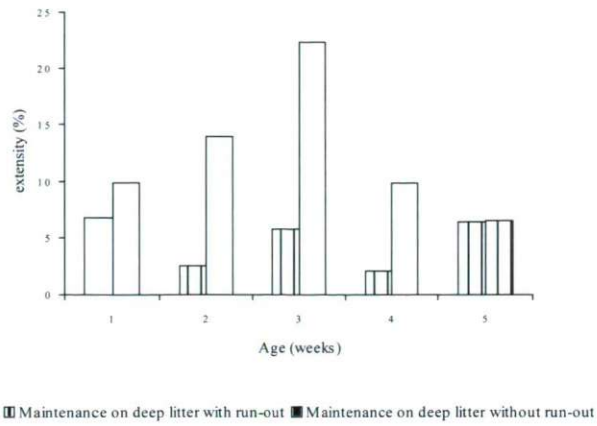

Figure 3. Age dependent extensity of Cryptosporidium spp. oocyst excretion of German Angus beef cattle calves maintained in housing on deep litter with (n = 50) and without run-out (n = 73) (Jäger et al. 2005).

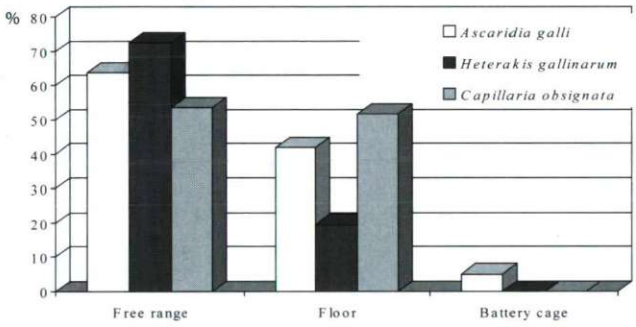

Figure 4. Prevalence (%) of gastrointestinal helminths in relation to production system (Permin et al. 1999).

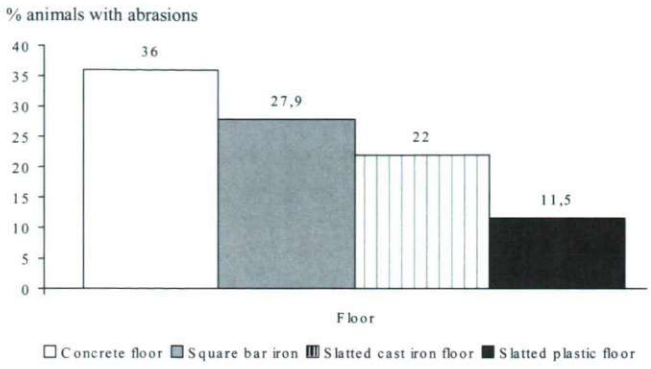

Figure 5. Frequency of intense abrasions in nursery pigs caused by floor system (Hoy 2003).

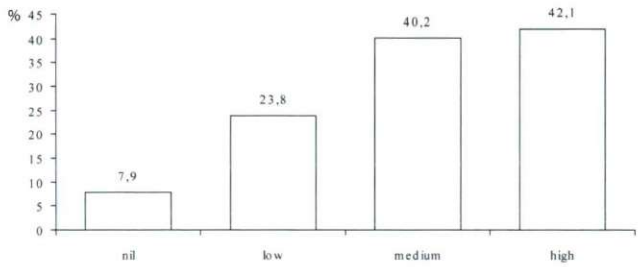

Figure 6. Relationship between degree of abrasions in nursery pigs and the frequency of arthritis treatments (Hoy 2003).

cow access to surface water,

disposal of manure in liquid form on land and

eating or grazing roughage by cows from fields having surface application of manure during the growing season.

Factors not associated with increased odds for *Samonella* shedding were herd size and farm type (organic/conventional). The authors concluded that management factors do influence the prevalence of *Salmonella* and therewith food safety.

Shitandi and Sternesjo (2004) evaluated the prevalence of multidrug resistant Staphylococcus aureus in Kenyan milk and investigated the differences in antimicrobial resistance between large- and small- scale producers. Susceptibility profiles for penicillin G, tetracycline, erythromycin, trimethoprim/sulfamethazine, and chloramphenicol were determined for *Staph. aureus* (n = 402) isolated from cows with subclinical mastitis. There was a significant difference in the overall mean resistance profile between large- and small-scale farm isolates. The overall prevalence of multidrug resistance differed significantly between isolates from small (34.3%) and those from large farms (18.0%). Additionally, the producers were interviewed about their usage of antimicrobial drugs and their attitudes towards education in related fields. There was an evident difference between the producer types in their documentation of the use of antimicrobial drugs. Small-scale farms were less inclined to documentation, and treatment records were available from 22% of the small-scale farms, compared with 73% of the large-scale farms. Farmers expressed a need for more information in 5 areas, ranking preventive management highest; followed by affordable tests to control residues in milk; preparation of antimicrobial drugs; public health concerns; disposal of surplus antimicrobial drugs; and antimicrobial drug residue persistence in milk. It was concluded that herd size might be an indirect risk factor in the development of antimicrobial resistance in Staph. aureus within the region. The results further suggested that lack of understanding of risks related to antibiotic contamination of food, poor or no treatment records, and lack of a monitoring system were the major risks for contamination. In conclusion the education among dairy producers greatly affects the occurrence of antimicrobial residues in milk (Shitandi and Sternesjo 2004).

Table 3. Antibiotic residues in eggs in relation to management system (Hafez et al. 1988; Friedrich et al. 1985).

Antibiotic, concentration	Days of treatment	Residues in egg after treatment (days) Cage	Floor system
Nicarbazin (2 mg/kg Futter)	29	16	> 60
Tetracyclin (500 mg/l water)	7	26	37
Enrofloxacin (50 mg/l water)	4	8	> 46

Table 5. Effects of cleaning and disinfection on performance and health in pigs (Anonymous 1998).

	Cleaning and disinfection yes	Cleaning and disinfection no
Farms (%)	47.9	52.1
Daily weight gain (g)	683	649
Costs for vet and disinfection/animal (€)	0.97	0.94
Mortality (%)	3.08	3.4

Management systems and food safety in chickens

It has been proved in many systems that excellent hygiene management can significantly lower the use of drugs. In industrialised countries most laying hens are kept in cages where they are separated from their faeces. Faeces are the main source of many infections. As a result the prevalence and economic importance of infections in chickens has been very low in recent decades (Permin et al. 1999). Besides positive hygiene effects, cages had other major advantages like economical factors, productivity (eggs, food conversion rate etc.), less stress for the animals because of smaller group sizes, less bird aggression and cannibalism and low mortality rates. The major disadvantage of cages is that the expression of some essential behaviour forms is limited or not possible including nesting behaviour, dust bathing and stretching. Therefore lately, animal welfare issues and changes in consumer demands have resulted in new upcoming EU-regulations. These include a ban of conventional cages after the year 2012. Only so called enriched cages (claw abrasives, nests, dust bathes, perches) and floor housing systems will be allowed in most European countries (Germany will ban all forms of cages) after the new regulations. The use of floor systems will result in a renewed importance of different diseases. It was demonstrated by various authors that in floor systems there is an increase of bacterial and viral diseases (Table 2), parasite infections (Figure 4), mortality, total drug treatment, dirty and cracked eggs and chemical residues in food products (Table 3) as well as in the environment. Therefore floor systems are negatively correlated with food safety!

Table 4. Effects of vaccination against Mycoplasma hyopneumoniae on fattening performance in pigs (Welp et al. 1999).

Vaccination	Yes	No
n =	12.000	20.000
Daily weight gain (g)	697	682
Feed conversion rate 1 :	2.97	3.03
Mortality (%)	2.54	3.54

Management systems and food safety in fattening pigs

Hoy (2003) showed that the frequency of intense abrasions in nursery pigs are related to the quality of floor systems (Figure 5), which is correlated with the frequency of arthritis treatments needed for the subsequent life of the animals (Figure 6).

The major ways to improve hygiene on pig farm level are vaccinations, all in-all out management, cleaning and disinfection and the control of the transfer of animals, staff ,feed stuff etc.. The effects of vaccination against Mycoplasma hyopneumoniae on fattening performance in pigs and the effects of cleaning and disinfection on performance and health in pigs are shown in tables 4 and 5. It was clearly shown that housing and management influence pig health, which is directly correlated with food safety!

Overall conclusions

Production and management systems do significantly effect animal health and therewith the use of drugs and prophylactic treatments. This may lead to residues and a change for the worse in food safety.

The consequent implementation and use of hygiene and management tools and measures is needed, as well as a combination of these measures with effective programmes (e.g. vaccination, procedure with cleaning and disinfection, all in all out etc.) in order to limit the use of drugs in sick animals so they are used only when absolutely necessary. A better education among producers is also an important step towards higher food safety on farm level.

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ARTICLE

Knowledge and acceptance of genetically modified foodstuffs in Hungary

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ABSTRACT The safety evaluation of genetically modified (GM) foodstuffs is a highlighted research topic. European consumers are cautious with GM plants, their release into the environment and the consumption of GM foods. Technological changes and achievements are more and more difficult to be understood for consumers. Novel technologies and the products of the biotechnology industry are thought to bring additional risks into consumers' life according to their perception. Consumers perceive risks on a different way than experts. 556 respondents were involved in the first survey focusing on food safety than 1000 respondents were involved in the questioning survey intending to reveal consumers' knowledge and opinion about GM products and techniques. The opinion of consumers and professionals about gene technology is mostly negative as far as 35% of the consumers can recall more negative than positive information about GM foodstuffs and 13% can recall only negative ones. Nevertheless even if Hungarian consumers predominantly refuse GM products this proportion is still much smaller than in Western-Europe. According to 73% of the respondents it is essential to indicate the GM content on the packaging. Consumers are not sufficiently aware of the concept of biotechnology and often misunderstand it. The results reflect the insufficient information level of the Hungarian consumer and the misunderstanding of biotechnology concept.
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KEY WORDS

food
GMO
safety

The modification of the genetic structure in agricultural raw materials and foodstuffs is one of today's most debated issues and one of the most controversial research areas. On the one hand a large number of arguments have been mentioned concerning the economical and environment friendly nature of genetically modified (GM) products. Increasing attention is paid to food related risks and to the environmental impact of human activities and the application of scientific achievements. The safety evaluation of GM foodstuffs is a highlighted research topic. Many international organizations are involved in the risk assessment and safety evaluation of GMOs working out relevant methods and principles. Meanwhile consumers – particularly the European ones – are cautious with genetically modified plants, their release into the environment, the consumption of GM foods and other novel technologies, too.

Technological changes and achievements are more and more difficult to be understood for the consumers. Novel technologies and the products of the biotechnology industry are thought to bring additional risks into consumers' life according to their perception. Consumers perceive risks on a different way than experts. Experts believe that certain chemical and physical risks are far less disquieting than for instance biological, especially microbiological risks. Moreover certain physical food preservation methods – including irradiation – are considered much safer – due to the lack of residues – in terms of consumer health than chemical preservation, but this view hasn't been accepted by the consumers. Customers' decision concerning the purchase of foodstuffs is not primarily influenced by the latest scientific results but by several other socio-economic, emotional, political, ethical, environmental factors. According to experts and the surveys carried out in this field irradiation of foodstuffs should have become a widely used physical preservation technique. However, due to emotional reasons and lack of appropriate information, consumers haven't accepted it, and what is more in many cases they definitely refused this way of food preservation.

May the appearance and acceptance of GM plants and crops face similar consumer distrust in European markets as well? Why are consumers so suspicious about GM products? Do Hungarian consumers possess appropriate information about GM foodstuffs? What is the evaluation of genetically modified foodstuffs like (consumers recall positive or negative information and news)? Which products (traditional or GM ones) are preferred by domestic consumers? Are consumers aware of the meaning of genetically modified foodstuff? To what extent are consumers concerned about the safety of GM foodstuffs? Should it be indicated on the label that the given product contains GM ingredient?

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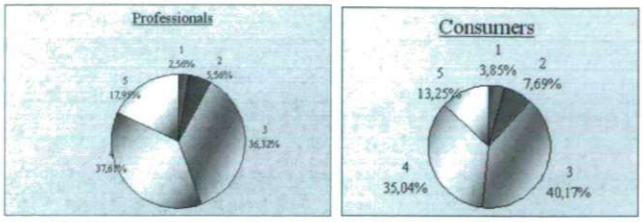

	1	Predominantly positive
	2	Rather positive than negative
	3	Mixed or neutral
	4	Rather negative than positive
	5	Predominantly negative

Figure 1. Evaluation of genetically modified foodstuffs.

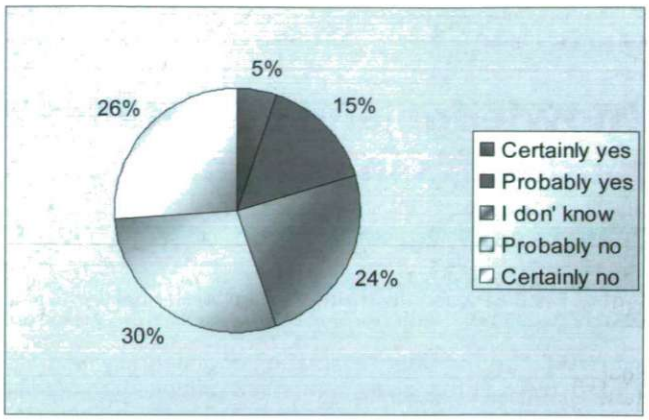

Figure 2. Selection choice between foodstuffs made of GM and traditional raw materials.

We have been looking for the answers for the abovementioned questions within the framework of the recently established consumer science and consumer risk perception surveys in Hungary. The study of consumer risk perception and factors influencing risk communication is a part of our contribution to the establishment of the national food safety strategy.

Our research is made up of two major steps. First a general food safety questionnaire was compiled in order to reveal the differences between consumers and professionals. This questionnaire contained a distinct chapter with questions on genetically modified products. The results reflect the opinion of the Hungarian influential middle class. Following the results of the preliminary studies, in the next step only GMO related questions were asked. Altogether 556 respondents were involved in the survey. 256 persons out of them possess university degree in food industry and food science. 83.7% of the respondents have heard about the genetic modification of foodstuffs, which is good compared with the European average. The opinion of consumers and professionals about gene technology is mostly negative (Figure 1.). 35% of the consumers can recall more negative than positive information about GM foodstuffs; meanwhile 13% can recall only negative ones. Considerable number (40.17%) of the respondents remember news and information with neutral content, which largely influence their approach to the topic. Relatively a small proportion of consumers can recall more positive than negative (7.7%) or mainly positive (3.85%) information.

In the case of professionals the proportion of those, that recall mostly negative (17.95%) or more negative (37.61%) information is bigger. Therefore there is a significant difference in this respect between expert and non-professional respondents. The proportion of "neutral" answers is the largest in both groups.

Respondents then were asked to choose between two products, one of which contains theoretically GM ingredient, but possess better taste, appearance, lower price and longer shelf life than the traditional one. The analysis of the replies (Figure 2.) shows that even if the majority still refuses (51%) the number of uncertain opinions decreased by 15%. Consequently some 15% of the respondents could have been convinced with the advantageous properties of the GM product.

Altogether it can be stated that even if Hungarian consumers predominantly refuse GM products this proportion is still much smaller than in Western-Europe. Those GM products that possess better properties than the traditional ones are not preferred either, as only 5% of the respondents gave unambiguous yes answer for the above question. One fifth (20%) of the respondents would choose the genetically modified product if had the opportunity to select. This part of the survey outlines that far-reaching conclusions - concerning the refusal of GMOs - cannot be drawn from the emotional replies given to general questions. Since GM products are more accepted if they offer particular advantages. The same principle was seen during the selection between traditional and genetically modified animals, with the latter possessing better features. Therefore it is easy to understand why the acceptance of GM cereals – which appeared in public production in 1996 - is so low, as these crops possessed benefits (e.g. bigger yield and thus bigger profit) mainly for producers and not really for consumers. However the appearance of second and third generation GMOs may result in better acceptance. Then consumers were asked whether they expect distinct labelling of foodstuffs containing genetically modified ingredients (Figure 3).

According to 73% of the respondents it is essential to indicate the GM content on the packaging. Further 25% answered that it would be interesting to know and 2% replied

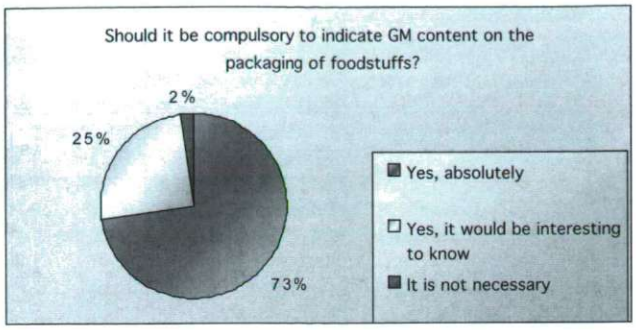

Figure 3. Opinions concerning the distinct labelling of GM foodstuffs.

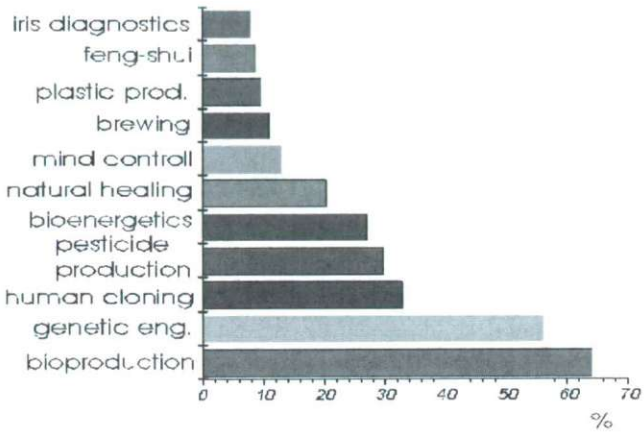

Figure 4. Interpretation of the biotechnology concept.

that it is unnecessary to mark GM content on the label. These answers confirmed the outstanding and increasing importance of foodstuffs' labelling from customers' point of view.

There are often positive and negative information spread about GMOs, which quite often has an impact on consumers' emotions. Some of them is to prove the essential nature of GMOs, meanwhile others emphasize risks from nature and consumer point of view. We studied on a 1-5 Linkert-scale how the respondents agree with the most frequently mentioned opinions on genetically modified plants. During the preparation of the questionnaires the traditional 1-5 scale was chosen as it is well known from the national school evaluation, furthermore it enabled the subsequent mathematical-statistic process. Table 1 shows that the biggest concern (3.93) is the disturbance of the natural balance and biodiversity. Concerns about the potential harmful effects on the human body are also significant (3.79). Consumers do not believe that GM plants are the ultimate solution for the feeding problems of the world's increasing population.

1000 respondents were involved in the questioning survey intending to reveal consumers' knowledge and opinion about

Table 1. Opinions on genetically modified products.

Statement	Average score
GMOs can disturb the natural balance and biodiversity	3,93
GMOs can damage our body	3,79
We mustn't intervene in God's work / in Nature	3,24
These are important in order to decrease the use of insecticides	3,01
GMO is the solution for poor countries struggling with starvation	2,93
It is important for foodstuffs with better taste and composition	2,81
More and more people need to be fed	2,40
GMOs are reliable as these were preceded by scientific experiments	2,38

GM products and techniques. Consumers are not sufficiently aware of the concept of biotechnology and often misunderstand it. More than two third (64%) of respondents meant bioproduction and ecological farming as part of biotechnology, thus demonstrating total confusion of terminologies (Figure 4). A bit more than half of the respondents (55 %) considered the modification of plant and animal genetic material as part of biotechnology. Those involved in disciplines (e.g. food production, food distribution, healthcare, agricultural production) where the modification of the genetic material is applied in the practice were of course more informed about the topic. Only 10% of the respondents considered brewing as a biotechnological method. Approximately the same number of respondent replied that feng shui, the ancient Chinese art and iris diagnostics, a way of natural healing are related to biotechnology.

The results reflect the insufficient information level of the Hungarian consumer and the misunderstanding of biotechnology concept. Those committed to modern biotechnological methods often refer to surveys according to which consumers answer "no" to the following question "Would you consume foodstuffs that contain DNA?" Thus justifying why energy and attention shouldn't be paid to incompetent consumer opinions and expectations. However consumer uncertainty and ignorance can be understood if we take the fact into consideration that DNA was discovered only a few decades ago and this discipline has been developing enormously. Consumers belonging to the older generation didn't have the opportunity either to learn about DNA or about the results of modern biotechnology and molecular genetics at school. Only some one third (34.5%) of those possessing university degree consider their biology and biotechnology knowledge as sufficient. Similar number of people believe they have basic knowledge in this field. Neither those having primary school nor those having secondary school (48.6%) qualification think they possess sufficient amount of information about the topic

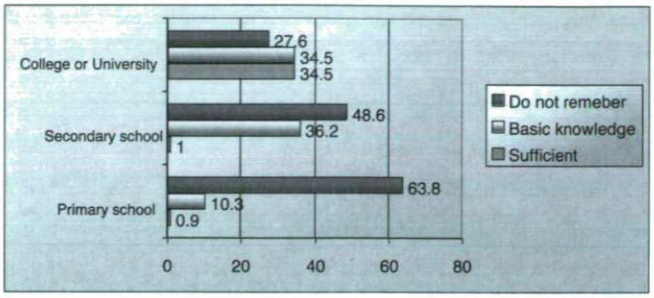

Figure 5. Correlation between the school related studies and knowledge of biotechnological results.

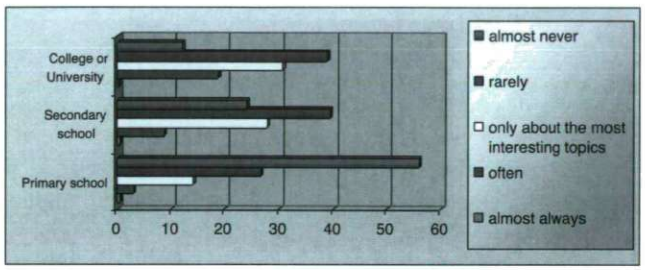

Figure 6. The relation of biotechnology as a conversation topic and qualification.

(Figure 5). One third of those with university qualification consider themselves appropriately trained and they believe that learned the basic principles. About half of those having secondary school qualification and 64% of those possessing primary school qualification replied that they didn't remember studies related to the topic.

The appearance of modern biotechnology in interpersonal communication as a conversational topic (Figure 6) largely depends on the qualification. Only 30.6% of those having university degree initiate conversation about the interesting relations of biotechnology, meanwhile 38.8% rarely mention the issue.

Respondents attributed similar roles to food control authorities, consumer protection NGOs, research institutes, universities and scientific associations in the protection of consumer interest. The trust towards the actual government and the press is significantly different. The results thus obtained a bit differ from the "trust index" experienced during other food safety surveys. In previous questioning research studies consumers unambiguously referred to independent researchers, research groups, the Hungarian Scientific Academy and the Central Food Science Research Institute as the most reliable organizations in the field of food safety. In the case of genetically modified foodstuffs, respondents probably feel that food control authorities should urgently take consistent steps in order to protect consumers' interest.

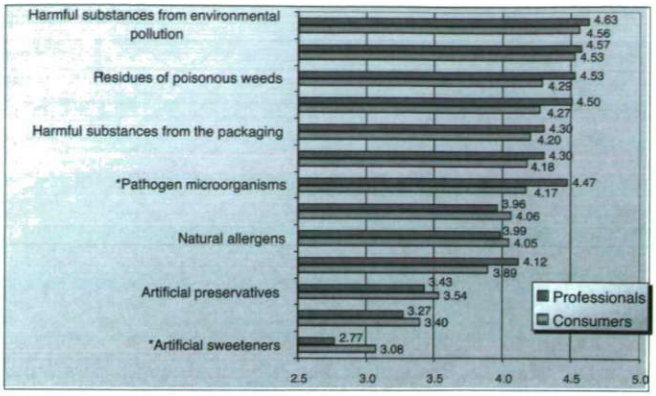

Figure 7. Risks attributed to genetically modified foodstuffs compared to other risk factors.

During the investigation of factors determining and threatening food safety (Figure 7) one might conclude that genetic modification was not considered among the most dangerous factors. In this respect there was no significant difference between the opinion of professionals and consumers. Both groups believed that harmful substances resulting from environment pollution, agricultural chemical residues, harmful substances dissolving from the packaging and pharmaceutical residues in meat are more dangerous. Mycotoxins, pathogenic microorganisms and poisonous weed residues were also considered more risky but concerning these latter factors there were significant differences between professional and consumer opinions. Regarding all above factors professionals reckoned them more dangerous than consumers. On the other hand consumers considered genetically modified foodstuffs, natural allergens, artificial preservatives, other additives and artificial sweeteners more risky than professionals did.

The majority of Hungarian consumers – just like EU consumers – refuse the genetic modification of plants and food raw materials. Concerning GMOs they recall rather negative than positive information and substantially agree with frequently mentioned statements about natural damages and threatening of human health. Although the evaluation of GMOs is basically not so favourable from professional and consumer viewpoints as well, they are less refusing than it is experienced in old EU member states. The number of uncertain consumers significantly decreases if the GM foodstuff offers advantageous properties to the consumer compared to the traditional one.

The evaluation and acceptance of GMOs may be influenced by biological and biotechnological awareness, knowledge and appropriate information. Consumers are not provided sufficient, processed and easy-to-understand information. The social dialogue concerning genetically modified crops and GMO containing foodstuffs is quite poor. Processing the information available exceeds the skills of

the average consumer. The development of biotechnology, molecular genetic knowledge and genetic engineering tools is faster than the codification or the establishment of legal and ethical norms.

The development of this discipline is far quicker than the widening of experts' knowledge. As the "biotech scissor" is opening there is an increasing difference between science, its practical applications and social judgement, acceptance. Information expected by consumers should immediately be supplied in proper and clear form. Regulations should be based on up to date scientific results of food safety research – taking into consideration the limitations of the means and knowledge available (e.g. application of the precautionary principle), consumer expectations and other legitimate factors influencing their decisions. Risk communication must be improved and based on the results of risk assessment and safety evaluation.

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This study was presented on the NATO Advanced Research Workshop on "Food Safety and Security" held between 13-15 September, 2004, at Lake Issyk-Kul, Kyrgyzstan. The workshop was funded by NATO. Co-directors were Prof. Dr. A. Aldashev, National Academy of Sciences of the Kyrgyz Republic, and Prof. Dr. L. Erdei, University of Szeged, Szeged, Hungary.

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ARTICLE

Variation of ABA and carotenoids during flowering initiation in carrots

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ABSTRACT The aim - to investigate the dynamics and interaction of abscisic acid and carotenoids in carrot during different flowering initiation stages. The process of carrot flowering initiation and morphogenesis was studied in a phytotron facility: EXP1 – photoperiod of 0 h and 4°C temperature, EXP2 – photoperiod of 8 h and 4°C temperature, EXP3 – photoperiod of 16 h and 4°C temperature, EXP4 – photoperiod of 8 h and 21/16°C temperature, EXP5 – photoperiod of 16 h and 21/16°C (day/night) temperature. High-performance liquid chromatography (HPLC) with diode array detector was used for separation and determination of phytohormones (cytokinin, gibberellic acid, indol-3-acetic acid, and abscisic acid). Spectrofotometric analysis of total carotenoids quantification was made by 644 nm spectrophotometer. The development of carrots with 9 leaves in rosette in different treatments was not identical. Carrots grown in higher temperatures contained a higher level of carotenes in roots than did carrots grown in lower temperatures. Thought such tendencies were not observed in carrot leaves. During flower initiation stage the increase of ABA level in leaves and the decrease in roots was observed in all treatments. The common tendency of carotenoid content variation was observed under treatment with low temperatures and short day photoperiod. These changes can be connected with plant preparation for flowering and seed formation. To summarize, low positive temperatures and short day photoperiod conditioned the most rapid formation of elements of inflorescence axis and the fast growth of generative organs. Also these conditions determined low ABA levels in carrot leaves. Temperature makes the highest effect on carotenoid synthesis in carrot root although in leaves this effect is suppressed by short day photoperiod.
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KEY WORDS

abscisic acid
carotenoids
carrot
evocation

There exist several hypotheses, concepts and theories for explaining the mechanism of plant transition to the generative development (Chailakhyan 1988; Jordan 1993). In most of these theories, various aspects of the hormonal regulation of flowering are analyzed. It has been proposed that phytohormones are involved in the metabolism pathway, which occurs after light perception and transduction of the initial signal into a physiological effect (Jordan 1993). The carrot plant flowers only after vernalization, *i.e.*, low temperatures are needed for flower initiation (Kraepiel and Miginiac 1997). Reproductive success of plant largely depends on the correct timing of floral induction. This is the reason why the initiation of flowering is highly regulated by environmental cues exhibiting regular seasonal changes, such as photoperiod and temperature, and by the developmental stage of the plants (Bernier et al. 1993). The end of the juvenile period is linked to plant's utter preparation for photo- and thermo induction processes. In carrot ontogenesis this moment corresponds with the beginning of carrot root thickening. At that moment plants form 8 assimilating leaves. Photo- and thermo induction mechanisms are not interrelated can proceed at different time and stipulate different effects (Duchovskis et al. 2003). According to P. Duchovskis, there are two periods in flowering induction and evocation. The first period of flowering induction is photo induction (5 leaves in rosette for carrot). Metabolites of photomorphogenetic system are transported to apical meristems, where they can de-block the genes of inflorescence axis formation. From this moment evocation period first starts which ends with the formation of the inflorescence axis. The second period of flowering induction of wintering plants is thermo induction (vernalization; 9 leaves in rosette for carrot); its metabolites determine the formation of inflorescence axis structures (evocation period second). These processes requite in flower initiation and differentiation (Duchovskis and Samuoliene 2004).

Abscisic acid (ABA) is a sesquiterpenoid plant growth regulator has multiple functions and is involved in many physiological and developmental processes such as transpiration, germination, dormancy, and adaptation to environmental stress (Rock and Zeevaart 1991). ABA seems to act as a general inhibitor of growth and metabolism, but these effects vary with tissue and development stage. ABA is present in many

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growing regions, and its levels do not seem to be reduced in actively growing buds. Nonetheless, ABA applied to hypocotyls, epicotyls, leaves, and coleoptiles is generally inhibitory to growth (Srivastava 2002). Carotenoids represent a diverse and a widely distributed class of pigments. They are synthesized de novo in all chlorophyll-containing photosynthetic organisms (Goodwin and Britton 1988). In plants, carotenoids are essential for photosynthesis (Demmig-Adams and Adams 1996); serve as the precursor for the biosynthesis of abscisic acid (Rock and Zeevaart 1991; Schwartz et al. 1997); and act as coloring agents. In photosynthetic tissues, carotenoids accumulate in the membranes of chloroplasts (Siefermann-Harms 1987), while in non-photosynthetic tissues they accumulate in chromoplasts (Camara et al. 1995). Chromoplasts frequently derive from fully developed chloroplasts, as seen in tomato (*Lycopersicon esculentum*) fruits. In other instances, chromoplasts can arise from non-photosynthetic plastids, as in carrot (*Daucas carota*) roots (Marano et al. 1993). In all cases, chromoplasts accumulate large amounts of carotenoids in specialized lipoprotein-sequestering structures (Vishnevetsky et al. 1999). In plants, carotenoid biosynthesis is a multifaceted and highly regulated process (Harker and Hirschberg 1998).

The aim of this study was to investigate the dynamics and interaction of abscisic acid and carotenoids in carrot during different flowering initiation stages.

Materials and Methods

Carrot *Daucus carota* L. var. *Garduolė2* was initially grown in vegetative tumbler 54x34x15 cm size placed in a greenhouse (16 h photoperiod and 21/16°C day/night temperature). The peat (pH ~6) was used as the substrate. Carrots with 9 leaves in rosette were moved to phytotron chambers with different conditions for 120 days: EXP1 – photoperiod of 0 h and 4°C temperature, EXP2 – photoperiod of 8 h and 4°C temperature, EXP3 – photoperiod of 16 h and 4°C temperature, EXP4 – photoperiod of 8 h and 21/16°C temperature, EXP5 – photoperiod of 16 h and 21/16°C (day/night) temperature. After that organogenesis processes (Kuperman et al. 1982) were investigated with the photoperiod of 16 h and 21/16±2°C (day/night) temperature maintained. Plants were investigated under illumination using HPI-T lamps (Philips).

Samples for high-pressure liquid chromatography (HPLC) were prepared by grounding 1-2 g of fresh tissue per sample (from roots and leaves) into powder under liquid nitrogen treatment. The samples were pre-purified using solid-phase extraction with NH_2-cartridge columns. The prepared samples were stored in vials at 4°C, as proposed by Wang Y, 2003.

Analysis of abscisic acid (ABA) was performed using a Shimadzu HPLC model 10A chromatographer (Japan) equipped with DAD detector (SPD-M 10A VP), column oven (CTO-10AS VP), degasser (DGU-14A), and two pumps (LC-10AT VP) enabling use of concentration gradient of the mobile phase. Separation and detection were performed on an Inertsil ODS-2 column (150 x 4.6 mm^2). Mobile phase gradient of 55% methanol in 1% acetic acid for ABA was used. The wavelengths of 254 nm were set in the DAD detector for ABA detection. The total run time for the separations at a flow rate of 1 mL/min was approximately 10 min.

Spectrofotometric analysis (spectrophotometer Genesis 6, USA) of total carotenoids quantification was made by 644 nm spectrophotometer. 0.2g of fresh weight (from roots and leaves) was grounded with $CaCO_3$ and extracted in 100% acetone, according to Vetsthtein (Gavrilenko 1975).

The following chemicals were used: acetone(POCH, Poland), $CaCO_3$ (LaChema, Czech Republic), isopropanol (POCH, Poland), imidazole, ABA (Sigma-Aldrich, Germany), NH_2 -columns (Supelco, USA), methanol and hexane (LaChema, Czech Republic), acetic acid (BOH, England).

Results

The highest concentration of ABA was in carrot leaves before flowering induction and in roots it was very low (Fig. 1A and B). Whenever, such strong variation in carotenoids content was not observed (Fig. 2A and B).

Under treatment with photoperiod of 0 h and 4°C temperatures, the development of carrots stopped after evocation stage II. The concentration of ABA was low and almost equal in both leaves and roots during evocation period I and II (Fig. 1A and B). During evocation period I and II the concentration of total carotenoids was the same in leaves as before flowering induction, and decreased twice in carrot roots (Fig. 2A and B).

The development of carrots with 9 leaves in rosette in dif-

Table 1. The intensity level of carrot development processes during different flowering initiation stages.

Treatment		EXP1	EXP2	EXP3	EXP4	EXP5
Flowering initiation stage	Evocation stage I (organogenesis stage III)	+	+ + +	+ + +	+ +	+ +
	Evocation stage II (organogenesis stage IV)	+	+ + +	+ + +	+ +	+ +
	Flower initiation (organogenesis stage V^a)	-	+ + +	+ + +	+ +	+ +
	Flower differentiation (organogenesis stage V^b, V^c)	-	+ + +	+ + +	+ +	+ +

"+/-" - development intensity level in carrot.

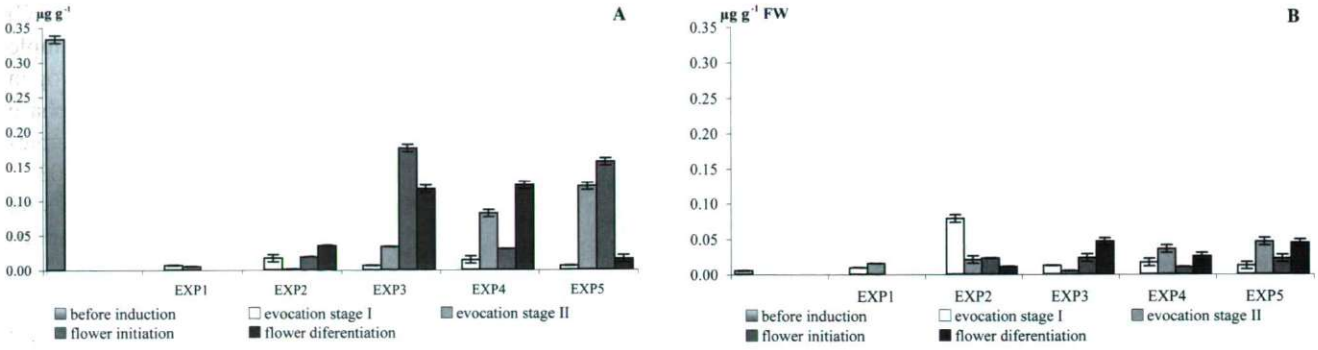

Figure 1. ABA content variation in carrot leaves (A) and roots (B) during various flowering initiation stages.

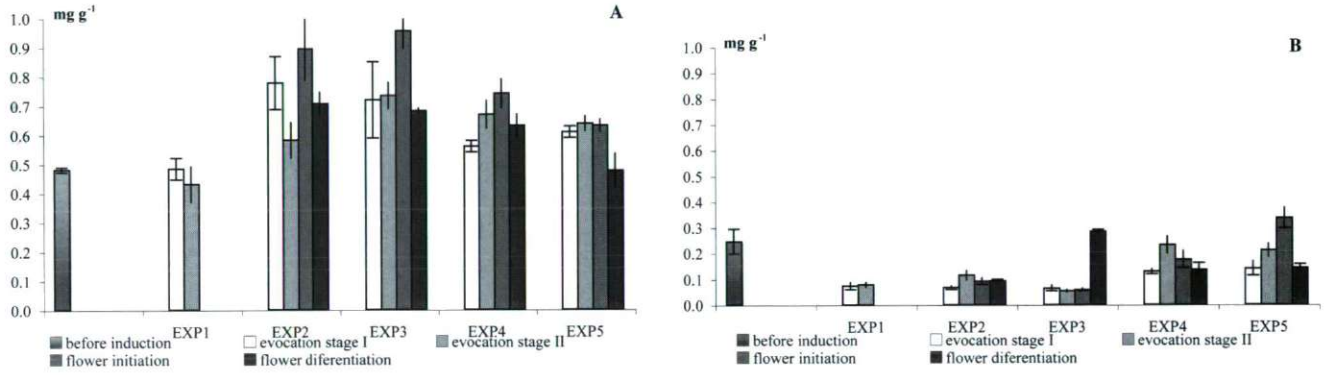

Figure 2. Carotenoids content variation in carrot leaves (A) and roots (B) during various flowering initiation stages.

ferent treatments was not identical. Elements of inflorescence axis were formed (organogenesis stage IV) and the growth of generative organs was the best in treatments EXP2 and EXP3 (Table 1). After flowering induction in evocation stage I, higher contents of ABA were accumulated in carrot roots than in leaves. But in both roots and leaves ABA content was lower (about 2.5 times in leaves) under treatment with long day (LD) than under treatment with short day (SD) photoperiod independently of temperature regime (Fig. 1A and B). And during this stage the highest concentration of ABA in carrot roots and leaves was detected under treatment with SD photoperiod and low temperatures (EXP2) which dramatically decreased during evocation stage II. It was observed the decrease of ABA concentrations in carrot roots during evocation stage II, flower initiation and differentiation processes. The lowest concentrations of ABA were detected in carrot leaves under SD and low temperature treatment (EXP2). The decrease in ABA content also is observed under treatment with high temperatures in flower initiation stage (EXP4) and in flower differentiation stage (EXP5; Fig. 1A).

Whenever, carotenoids concentrations were higher in leaves than in roots in all treatments during various flowering initiation stages. In carrot roots higher concentrations of carotenoids were detected under treatment with high (EXP4, EXP5) than with low positive temperatures (EXP2, EXP3) treatment during all flowering initiation stages (Fig. 2B). In leaves under treatment with low positive temperature (EXP2, EXP3) the total content of carotenoids increased during flower initiation stage, whereas under treatment with high temperature it was more stable (EXP4, EXP5; Fig. 2A).

Discussion

As it is known, different carrot development level is needed for photo and thermo induction (Duchovskis et al. 2003; Duchovskis and Samuoliene 2004). Photo- and thermo induction mechanisms are not interrelated. For the first stage of flowering induction (photo induction) 5 leaves in rosette are needed. From this moment starts evocation stage first which ends with the formation of the inflorescence axis. After that the second evocation stage (thermo induction) starts. At least 8 assimilating leaves are needed in order to react to thermo induction. During these processes the formation of inflorescence axis elements is complete (Duchovskis et al. 2003). The development of the investigated plants was unequal, the best development rate was under treatment with low posi-

tive temperatures and it lightly depended on the duration of photoperiod.

Environmental cues are perceived by different organs in the plant and promote endogenous stimuli that signal the apical meristem (Lejeune et al. 1991). Biennial plants cannot be vernalized as imbibed seeds or young seedlings but rather must reach a critical age or developmental stage before vernalization can occur (Lang 1986). The shoot apex must be exposed to cold for vernalization to occur. This is consistent with vernalization causing the apical meristem to acquire competence to flower. In our experiment carrots under treatment with low temperatures showed the more rapid development.

The major ecological function of the carrot root is as a reserve of assimilates for the production of a flowering stem after appropriate stimuli (Hole 1996). It is known that carotenoids are not widely distributed in root crops. Alpha and beta carotene accounts for more than 90% of all carotenoids in carrot (Simon and Wolff 1987). The carotenoid composition is enriched by the presence of biosynthetic precursors, because plants are able to synthesize carotenoids de novo. Carotenoids protect photosynthetic organisms against potentially harmful photo oxidative processes and are essential structural components of the photosynthetic antenna reaction center complexes. In plants, some of these compounds are precursors of abscisic acid, phytohormones, that modulates developmental and stress processes (Koornneef 1986). According to the currently accepted hypothesis, ABA form products of the oxidative cleavage of xanthophylls. Therefore, all steps involved C_{40} compounds are common to both carotenoids and ABA, whereas steps after xanthophylls cleavage are specific for ABA biosynthesis.

Nilsson (1987) found a strong positive correlation between carotene content and accumulated day-degrees above 6°C. Carrots grown in higher temperatures contained a higher level of carotenes than did carrots grown in lower temperatures. Also it is known that carotene content increases with the age and size of the root. These data also correlates with our results observed analyzing carotenoids in carrot root (Fig. 2B). Thought such tendencies were not observed in carrot leaves (Fig. 2A). Also it can be related with carotenoids most important function – the protection against harmful oxygen species in photosynthetic tissues. In nonphotosynthetic tissues, in the absence of carotenoids, plants also suffer severe photo oxidative damage, which generally results in the death of the organism.

The regulatory mechanism of ABA biosynthesis is subject to complex regulation during plant development and in response to environmental stresses (Xiong and Zhu 2003). In vegetative tissues, ABA levels increase when plants encounter adverse environmental conditions (low temperatures). Studies of abscisic acid biosynthesis suggested that ABA in higher plants is synthesized from an "indirect" pathway through the cleavage of a C_{40} carotenoid precursor, followed by a two-step conversion of the intermediate xanthoxin to ABA via ABA-aldehyde (Seo and Koshiba 2002; Schwartz et al. 2003). It was found that carotenoid-deficient plants are ABA-deficient (Quarrie and Lister 1984). The highest level of both carotenoid and ABA was detected in leaves under treatment with low positive temperatures and long day photoperiod during flower initiation stage (Fig. 1A and Fig. 2A). During this stage the increase of ABA level in leaves and the decrease in roots was observed in all treatments (Fig. 1A, B). The common tendency of carotenoid content variation was observed under treatment with low temperatures and short day photoperiod (Fig. 2A). These changes can be connected with plant preparation for flowering and seed formation.

To summarize, low positive temperatures and short day photoperiod conditioned the most rapid formation of elements of inflorescence axis (organogenesis stage IV) and the fast growth of generative organs. Also these conditions determined low ABA levels in carrot leaves. Temperature makes the highest effect on carotenoid synthesis in carrot root although in leaves this effect is suppressed by short day photoperiod.

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ARTICLE

Differential effects of hexaconazole and paclobutrazol on the foliage characteristics of Chinese potato (*Solenostemon rotundifolius* Poir., J.K. Morton)

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ABSTRACT In the present investigation, the impact of hexaconazole (HEX) and paclobutrazol (PBZ), triazole fungicides, on the leaf anatomy of Chinese potato (*Solenostemon rotundifolius* Poir., J.K. Morton) was studied. The thickness of leaf, upper and lower epidermis, number of palisade and spongy cells per unit area, number of chloroplast per palisade and spongy cells, number of stomata in upper and lower epidermis, stomatal pore length and width were observed in both control and treatments. Leaves treated with HEX and PBZ showed several variations in the anatomical characteristics. **Acta Biol Szeged 50(3-4):127-129 (2006)**

KEY WORDS

Solenostemon rotundifolius
hexaconazole
paclobutrazol
anatomy
stomata

Solenostemon rotundifolius Poir., Morton is one of the important vegetable crops belongs to the family Labiatae and cultivated in many parts of the world for its edible tubers. In the phylogenetic and taxonomic studies of the higher plants, anatomical characteristics have a profound role. The structure and ontogeny of stomata in different plants will vary with the application of different growth regulators (Gupta et al. 2004). Leaf anatomy is an important feature for internal water balance of the plants. The anatomical characteristics were found changed due to the application of growth regulators. Triadimefon treated mulberry plants showed great variations in the stomatal structure and functions (Sreethar 1991). Triazole compounds are systemic fungicides, which have plant growth regulating properties (Fletcher and Hofstra 1990).

The impact of triazole plant growth regulators on hormonal changes (Ye et al. 1995; Fletcher et al. 2000), photosynthetic rates (Panneerselvam et al. 1997) and enzyme activities (Muthukumarasamy and Panneerselvam 1997) have been reported. The plant growth regulating properties of triazoles are mediated by their inference with isoprenoid pathway and shift in the balance of plant hormones (Fletcher et al. 2000). Paclobutrazol (PBZ) increased the leaf thickness in rape plant due to elongated palisade cells (Zhou et al. 1993) and wheat leaves (Sopher et al. 1999). The triazole compounds protect plants from chilling stress (Feng et al. 2003), salt stress (Muthukumarasamy et al. 2000) as well as exhibit powerful fungicidal properties (Davis and Curry 1991). Previous works proved the ability of triazole compounds such as triadimefon (TDM) in enhancing the antioxidant potential in plants like *Catharanthus roseus* (Jaleel et al. 2006). The information available so far about the effect of triazole on leaf anatomy in plants is less. Hence it is aimed to understand the effect of triazole compounds such as hexaconazole (HEX) and PBZ in *S. rotundifolius*. The objectives of the present investigation were to study the impact of HEX and PBZ on leaf thickness, thickness of upper and lower epidermis, number of palisade and spongy cells per unit area, number of chloroplast per palisade and spongy cells and number of stomata and stomatal pore length in *S. rotundifolius* plants.

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Materials and Methods

The tubers of *S. rotundifolius* were obtained from Central Tuber Crop Research Institute (CTCRI), Kerala and planted in the Botanical Garden of Annamalai University, Tamil Nadu. In the present investigation, a field experiment was conducted in Randomized Block Design (RBD) with 7 replicates in *S. rotundifolius* during 2004-2005. Each plant was treated with 10 mg l^{-1} (active principles) of HEX and PBZ on vegetative stages like 80, 100 and 140 days after planting (DAP). The treatments were given by soil drenching. The fully expended mature leaves of plants, which emerged after the treatments were collected randomly on 90, 120 and 150 DAP from each concentration and control.

The leaves were washed thoroughly with water and fixed them in formalin: acetic acid: ethyl alcohol (5:5:90 v/v/v). Thin transverse-sections were taken, stained and observed under calibrated light microscope and the thickness of leaf was measured by precalibrated ocular micrometer. Epidermal peels were taken out from the basal, middle and apical regions by adopting direct peel method. The epidermal peels were

stained with 1% Delafield's haematoxylin and mounted in 50% glycerin (Dwivedi and Singh 1990). The observations were taken on 90, 120 and 150 DAP in seven replicate peels in each treatments. The leaf, upper and lower epidermis thickness expressed in micrometers and number of stomata in upper and lower epidermal cells per unit area were calculated by using the generally followed formula of Metcalfe and Chalk (1979). The length and width of stomatal pores were measured randomly in each treatment on the lower surface. The number of spongy cells per unit area and chloroplast per palisade and spongy cells were also calculated separately to find out the relative effect of triazole compounds.

Statistical analysis

The data were analysed using the analysis of variance (ANOVA) as described by the method outlined by Ridgman (1975). Means were compared between treatments from the error mean square by Least Significant Difference (LSD) at the $P \leq 0.05$ and $P \leq 0.01$ confidence level using Tuckeys (1953) test.

Results and Discussion

Thickness of leaves treated with triazoles was increased to a level higher than that of control leaves in *S. rotundifolius* plants. Among the triazole treatments there is no significant variation in thickness of leaf, upper and lower epidermis (Table 1). TDM treatments increased the thickness of leaf in plants (Asami et al. 2000).

The number of cells per unit area in the palisade spongy layers and chloroplast number per cells in the leaves increased by the HEX and PBZ treatments when compared to control leaves (Table 2). Among the triazole treatments, there was no significant difference in these characters. Increased mesophyll thickness, chloroplast size and level were reported in wheat with TDM (Gao et al. 1988). Triazoles increased the cytokinin levels in various plants like cucumber (Fletcher and Arnold 1986). The increased cytokinin level also can accelerate chloroplast differentiation and chlorophyll production and also protect the integrity of chlorophyll molecule (Fletcher et al. 2000).

Several variations like stomatal pore length, width and unequal accessory cells were observed in treated leaves. In the case of untreated leaves all stomata are open and have large stomatal pore length but width of stomata gradually decreased in the leaves of treated plants (Table 2). Triazole treatments caused the closure of stomata in bean (Fletcher and Hofstra 1988). Thiapenthenol reduced stomatal opening and reduced water consumption in mesophyll, a transient raise in the ABA content in bean (Asare-Boamah et al. 1986). This increased ABA content might have induced the stomatal closure as observed in uniconazole treated *Phaseolus vulgaris* (Mackay et al. 1990).

From the above observations it is clear that the triazole compounds affected stomatal pore length and width, stomatal pore size, thickness of upper and lower epidermis and the number of stomata, palisade, spongy cells, chloroplast per palisade and spongy cells. This is in accordance with the previous reports of Bora et al. (2002) and Gupta et al. (2004). It is previously reported that the application of PBZ can in-

Table 1. Effect of triazole fungicides on leaf anatomical characteristics of *S. rotundifolius*.

Growth stages (DAP)	Control	HEX 10 mg l^{-1}	PBZ 10 mg l^{-1}	LSD
	Leaf thickness (μ meter)			
90	62.18*	74.4*	66.13*	0.87
120	88.48*	89.40*	88.33*	1.24
150	89.61*	89.91*	86.15*	1.31
	Thickness of upper epidermis (μ meter)			
90	11.83*	14.36*	14.15*	0.67
120	12.24*	13.04*	14.31*	0.62
150	13.02*	16.03*	13.67*	0.58
	Thickness of lower epidermis (μ meter)			
90	8.43^{NS}	7.19^{NS}	7.11^{NS}	0.14
120	9.56*	8.94*	9.94*	0.12
150	9.99*	10.12*	9.99*	0.18
	Number of palisade cells per unit area			
90	21.54^{NS}	22.71^{NS}	21.61^{NS}	0.18
120	22.61*	23.41*	24.48*	0.20
150	23.13*	25.67*	25.01*	0.21
	Number of spongy cells per unit area			
90	37.04**	42.10**	41.41**	0.86
120	38.99**	45.61*	44.31*	0.94
150	41.67**	49.41*	49.01*	1.02

HEX – hexaconazole; PBZ – paclobutrazol; LSD – least significant difference; NS – non significant; *significant at 0.05 level; **significant at 0.01 level

Table 2. Effect of triazole fungicides on leaf anatomical characteristics of *S. rotundifolius*.

Growth stages (DAP)	Control	HEX 10 mg l^{-1}	PBZ 10 mg l^{-1}	LSD
	Number of chloroplast per palisade cells			
90	12.14*	13.11*	13.01*	0.18
120	14.15^{NS}	14.30^{NS}	14.90*	0.26
150	14.17^{NS}	14.20^{NS}	14.84*	0.22
	Number of chloroplast per spongy cells			
90	11.50^{NS}	11.36^{NS}	12.95*	0.14
120	12.51*	14.49*	14.34*	0.15
150	13.34*	15.42*	15.59*	0.28
	Number of stomata in lower epidermis (nos/mm^2 leaf area)			
90	10.61*	11.59*	11.65*	0.17
120	12.41**	15.34**	15.50**	0.21
150	14.41**	17.45**	17.20**	0.26
	Lower stomatal pore length (μ meter)			
90	12.04**	10.51**	10.35**	0.12
120	13.01*	12.34*	11.75**	0.16
150	13.99*	12.98*	12.90*	0.18
	Lower stomatal pore width (μ meter)			
90	3.41*	2.52*	2.63*	0.08
120	3.50*	2.93*	3.00*	0.09
150	3.67*	3.10*	3.21*	0.09

HEX – hexaconazole; PBZ – paclobutrazol; LSD – least significant difference; NS – non significant; *– significant at 0.05 level; **– significant at 0.01 level

crease the xylem water potentials (Thakur et al. 1998) and can increase the cytokinins under drought conditions (Zhu et al. 2004). The judicious application of triazole like HEX and PBZ may prove to be a useful tool for decreasing transpiration and inturn inducing drought avoidance mechanisms. It can be concluded that triazole such as HEX and PBZ may be useful to trigger drought avoidance mechanisms in plants like *S. rotundifolius*.

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ARTICLE

Effect of organic fertilizers combined with benzo (1,2,3) thiadiazole-7-carbothioic acid S-methyl ester (BTH) on the cucumber powdery mildew and the yield production

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ABSTRACT Organic fertilizers such as compost, compost tea and seaweed extracts (Algean) combined with benzo (1,2,3) thiadiazole-7-carbothioic acid S-methyl ester (BTH)) showed significant effect on the powdery mildew-infected cucumber leaves with Sphaerotheca fuliginea. We have shown that spraying the infected cucumber leaves with the BTH (0.05 mM) combined with the organic fertilizers strongly decreased the disease severity of the cucumber powdery mildew fungus from 85.1% to 3.4% as compared to the control leaves which infected only with the pathogen. Furthermore, organic fertilizers combined with BTH increased significantly vegetative growth characters of cucumber (stem length, number of leaves /plant, leaf area /plant and chlorophyll content) especially at the earlier stage of growth as compared to the control plants (chemical fertilizer only). Also, most of the organic materials produced the highest cucumber early yield and fruit quality, but total yield was equal or less than the chemical fertilizers. Interestingly enough, that organic fertilizers combined with BTH elevated the ascorbic acid content (chemical quality of cucumber fruits) and decreased the nitrate content which very harmful as well as increased the fruit yields as compared to the control plants.

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KEY WORDS

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Cucumber (*Cucumis sativus L.*) is a favorite commodity exports for markets and local consumption and represents one of the most important and economic vegetables in Egypt. It is grown in Egypt in the open field from March to November and under plastic houses from September to May. The total cultivated area of cucumber in Egypt was about 66640 feddan (6664 hectares) in 2005 according to the statistics of FAO.

Powdery mildew of cucumber caused by the fungus *Sphaerotheca fuliginea* (Schechtend Fr) Pollacci, is one of the most dangerous foliar disease, attacking cucumber plants, in Egypt and other countries (Harfoush and Salama 1992; Mosa 1997; Reuveni et al. 1997; Verhaar et al. 1997).

Fungicides and resistant or tolerant cultivars used to control this disease, however, each of these control methods has its limitations (McGrath 1991). Therefore, powdery mildews of cucumber plants (*Cucumis sativus* L.) remain the major problem for greenhouse producers worldwide.

The use of alternative control methods of diseases can effectively replace chemical fungicides. The application of safety chemicals to activate systemic acquired resistance (SAR-type reaction) provides novel alternatives for disease control in agronomic systems. Salicylic acid (SA) is the only plant-derived substance that has been demonstrated to be an inducer of SAR (White 1979; Antoniw and White 1980; Ward et al. 1991).

The synthetic chemical benzo (1,2,3) thiadiazole-7-carbothioic acid S-methyl ester (BTH) was also demonstrated to be a potent SAR activator (Friedrich et al. 1996; Görlach et al. 1996; Lawton et al. 1996) that supplies protection in the field against some diseases in several crops. Thus, BTH seems to be proper compounds for practical agronomic use (Hafez et al. 2004).

Geetha and Shetty (2002) found that chemical induction of resistance in pearl millet against downy mildew disease (*Sclerospora graminicola*) is possible by treating seeds of highly susceptible cultivars with the resistance activator benzothiadiazole (BTH) (CGA 245704), calcium chloride ($CaCl_2$) and hydrogen peroxide (H_2O_2). BTH in 0.75%, 90 mM $CaCl_2$ and 1.0 mM H_2O_2 were effective in managing the disease by giving 78%, 66% and 59% protection, respectively.

There is no doubt that chemical fertilizers are essential in most cropping systems. However, in long-term field experiments where mineral fertilizers have only been used, some problems could arise, especially increased soil erosion, soil compaction, environmental pollution and public health risk (Top et al. 2002). Therefore, it is essential to adopt a system of organic farming in vegetables due to increasing the objectives

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against the conventional farming as a main source of soil and water pollution as well as food products. As defined by the US Department of Agriculture (1980), organic farming is a system that excludes the use of synthetic fertilizers, pesticides and growth regulators.

Some investigators indicated that addition of organic manures as opposed chemical fertilizes increased vegetative growth characters, yield and fruit quality of vegetable crops (Ozoros-Hampton et al. 1994; Hsieh and Hsu 1995; Yousef et al. 2001; Poudel et al. 2002; Aly 2002).

On squash, Ozores-Hampton et al. (1994) showed that plants had increased yields when planted in municipal solid waste compost amended soil in spite of application of NPK fertilizers at recommended rates. On pepper, Hsieh and Hsu (1995) stated that early and total yields of all organic sources were significantly higher than that of chemical fertilizer. In the same line on cucumber, Aly (2002) found that organic treatment (compost) produced significantly greater early yield (1.85 kg/m^2) and total yield (4.49 kg/m^2) than chemical treatment which produced 1.38 kg/m^2 and 3.51 kg/m^2 for early and total yields, respectively.

Organic fertilizers are claimed to produce higher nutritional quality of vegetables in forms of vitamin C, TSS, dry matter and acidity (Vogtmann et al. 1993; Youssef et al. 2001; Bayoumi 2005). For nitrate content, Clark et al. (1999) found that nitrate content in tomato fruits was lowest in the organic system and highest in the conventional system as the differences were highly significant.

Hence, our investigation aimed to study the effect of BTH combined with compost with or without compost tea or seaweed extracts, on the growth, yield and fruit quality of cucumber crop comparing with the mineral fertilizers under plastic houses.

Materials and Methods

The experiments were carried out in the experimental farm of the Faculty of Agriculture, Kafr El-Sheikh University, Egypt during the winter season of 2005 and the early summer season of 2006 using cucumber hybrid (Prince) under plastic houses. Seedlings were transplanted on October 3rd (winter season of 2005) and February 5th (early summer season of 2006) on one side of the ridge (6 meters in length and 1 meter in width) at spacing of 30 cm between plants within the row. Plant density were 3.33 plants per square meter. Surface irrigation method was used.

Cucumber plants were infected with powdery mildew (*Sphaerotheca fuliginea*) spores naturally under greenhouse conditions. The control plants were heavy infected naturally.

The synthetic chemical benzo (1,2,3) thiadiazole-7-carbothioic acid S-methyl ester (BTH) in 50% sprayed to cucumber leaves in different concentrations (0.05, 0.075 and 0.1 mM). BTH sprayed three times on cucumber in the ages (30 days seedlings after transplanting immediately, 7 and 15 days after transplanting).

The experiment in each season included five organic and mineral treatments as follows:

1. Organic manure (compost) with 0.05 mM of BTH: Compost was added at the rate of 5 kg/m^2. Chemical analysis of compost was estimated immediately before its application (Table 1).

2. Compost accompanied with compost tea + 0.05 mM of BTH: Compost tea was a tea made from compost and water by soaking compost in water (1:1 v/v), it sprayed directly on the plants and applied also to soil with irrigation 5 times at fortnightly interval, starting three weeks after transplanting.

3. Compost combined with seaweed extracts (Algean) + 0.05 mM of BTH: Algean is a biological fertilizer, contains appreciable quantities of nutrients, hormones, amino aids and vitamins. It was used a foliar spray (2 ml/L) five times at two weeks intervals, starting three weeks after transplanting.

4. Mineral fertilizers with 0.05 mM of BTH: the recommended NPK fertilizers were used according to the recommendation of Ministry of Agriculture in Egypt.

Mineral fertilizers alone (control), as mentioned above without BTH.

Data recorded:

1- Disease severity % of the cucumber powdery mildew fungus.

2- Vegetative growth: stem length (cm), number of leaves/plant and leaf area/plant (dm^2) were determined at 45 and 60

Table 1. Chemical properties of compost used in 2005 and 2006 seasons.

Chemical analysis		Seasons	
		2005	2006
EC (dSm^{-1})		4.1	4.1
pH		7.5	7.6
O.M	(%)	33.9	32.7
Moisture	(%)	25.6	22.9
N	(%)	1.71	1.69
P	(%)	0.91	0.94
K	(%)	1.40	1.23
Fe	(ppm)	3380.4	3845.6
Zn	(ppm)	250.5	296.1
Mn	(ppm)	501.1	448.3

Table 2. Effect of BTH on disease severity percentage of cucumber powdery mildew.

Treatments	disease severity percentage %	
	2005 season	2006 season
Control	85.1	75.4
BTH 0.05 mM	3.4	4.4
BTH 0.075 mM	5.6	4.8
BTH 0.1 mM	9.8	10.88

days after transplanting.

3- Chlorophyll content in the leaves: relative green colour of one most recently matured leaf per plant was measured with SPAD meter (Minolta Corp, Ramsey, N.J.) after 45 and 60 days from transplanting.

4-Fruit yield: early yield was considered as the number and weight of fruits per square meter of the first four pickings. Total yield was determined as number and weight of fruits /m^2 of all pickings.

5-Fruit chemical quality:

a. Total soluble solids (TSS%): TSS % in juice of cucumber fruits was estimated by a hand refractometer according to A.O.A.C. (1965).

b. Ascorbic acid content (mg/100 g f. wt). It was estimated by titration with 2, 6-Dichlorophenol blue according to A.O.A.C. (1965).

Nitrate content (ppm): It was estimated by rapid colorimetric determination in fruits by nitration of salicylic acid according to Cataldo et al. (1975).

Experimental design and statistical analysis:

The experiment included five treatments, which were arranged in a randomized complete block design with three replications, as the treatments were distributed at random in the plots. Data were tested by analysis of variance (Little and Hills 1972). Duncan's multiple range test was used for comparison among the treatment means (Duncan 1965).

Results and Discussion

1. Effect of BTH on the disease severity % of the cucumber powdery mildew

When we sprayed the cucumber leaves with BTH in different concentrations (0.05, 0.075 and 0.1 mM), we found all the concentration increased the resistance against the powdery mildew. However, BTH in 0.05 mM was the best. BTH (0.05 mM) was able to decrease the disease severity from 85.1% to 3.4% in the both seasons 2005 & 2006 (Table 2).

Effect of organic fertilizers and BTH on vegetative growth characters and early and total fruit yields of cucumber

2-Vegetative growth

Data in Table 3 showed that stem length in cucumber plants was significantly affected by applying the different organic and mineral treatments at the two sampling dates (45 and 60 days after transplanting) in both seasons.

Applying the combination of compost + seaweed extract + 0.05 mM of BTH treatment (Tr.) No. 3, produced the long plants having both highest number of leaves and largest leaf area at 45 days after transplanting followed by Tr. No. 2 (compost + compost tea + 0.05 mM of BTH). Tr. No. 1 (compost + 0.05 mM of BTH) or Tr. No. 4 (mineral fertilizers + 0.05 mM of BTH) in both seasons. On the other side, Tr. NO. 5 (chemical fertilizers alone) produced the lowest values of stem length, number of leaves and leaf area/plant at the first stage in both seasons.

Dealing with the second sampling date, the results were varied compared to that of the first date, though Tr. No. 4 (Mineral fertilizers with 0.05 mM of BTH) produced the highest values of growth characters followed by Tr. No. 3 (compost + seaweed extract + 0.05 mM of BTH) or Tr. No. 5 (mineral fertilizers alone), Tr. No. 2 (compost + compost tea + 0.05 mM of BTH) and finally Tr. No. 1 (compost + 0.05 mM of BTH) which gave the lowest values of such vegetative growth parameters.

Table 3. Effect of organic and mineral treatments on some vegetative growth characters of cucumber plants in 2005 and 2006 seasons.

Treatments	Stem length (cm)		No. of leaves/plant		Leaf area (dm^2/plant)		SPAD green colour reading	
	Days after transplanting							
	45	60	45	60	45	60	45	60
	2005 season							
1. Compost + BTH	62.2 b	83.4 d	16.3 b	16.9 d	17.04 b	20.87 c	44.1 b	37.4 c
2. Comp. + Comp. tea + BTH	72.6 a	97.2 c	18.0 ab	22.6 c	20.87 a	24.67 ab	45.8 a	42.7 b
3. Comp. + Seaweed ext. + BTH	73.4 a	100.5 b	19.8 a	23.6 bc	21.30 a	24.96 ab	46.4 a	45.3 a
4. Mineral fertilizers + BTH	62.7 b	105.3 a	15.1 b	26.7 a	16.93 b	25.07 a	42.1 c	46.5 a
5. Mineral fertilizers alone (control)	56.0 c	100.5 b	15.0 b	24.2 b	16.66 b	24.2 b	41.9 c	37.1 c
F-test	**	**	**	**	**	**	**	**
	2006 season							
1. Compost + BTH	77.3 c	95.8 d	18.0 b	20.2 c	20.01 b	23.91 c	47.3 b	38.4 d
2. Comp. + Comp. tea + BTH	84.8 a	106.9 c	19.8 a	26.9 b	23.31 a	26.01 b	50.6 a	47.3 b
3. Comp. + Seaweed ext. + BTH	84.9 a	110.3 b	20.1 a	29.9 a	23.54 a	27.95 a	50.1 a	49.1 ab
4. Mineral fertilizers + BTH	79.9 b	114.5 a	17.8 b	30.2 a	20.05 b	28.18 a	44.6 c	50.5 a
5. Mineral fertilizers alone (control)	68.2 d	110.1 b	17.2 b	27.2 b	18.81 c	25.40 b	42.8 d	39.9 c
F-test	**	**	**	**	**	**	**	**

Means designed by the same letter are not significantly different at the 5% level according to Duncan's test.

Table 4. Effect of organic and mineral treatments on early and total fruit yields of cucumber plant in 2005 and 2006 seasons.

Treatments	Early yield No. of fruits/m²	Kg/m²	Total yield No. of fruits/m²	Kg/m²
	2005 season			
1. Compost + BTH	9.2 b	0.82 bc	28.5 d	2.20 c
2. Comp. + Comp. tea + BTH	11.6 a	1.00 b	36.9 b	2.21 c
3. Comp. + Seaweed ext. + BTH	11.6 a	1.67 a	36.5 b	3.14 b
4. Mineral fertilizers + BTH	7.8 c	0.69 c	38.9 a	3.53 a
5. Mineral fertilizers alone (control)	7.3 d	0.64 c	33.5 c	2.89 b
F-test	**	**	**	**
	2006 season			
1. Compost + BTH	10.9 b	1.06 b	32.8 d	2.65 d
2. Comp. + Comp. tea + BTH	12.8 a	1.49 a	40.1 b	3.60 b
3. Comp. + Seaweed ext. + BTH	13.0 a	1.47 a	43.0 a	4.09 a
4. Mineral fertilizers + BTH	9.9 c	0.85 c	44.2 a	4.05 a
5. Mineral fertilizers alone (control)	9.1 c	0.82 c	37.8 c	3.14 c
F-test	**	**	**	**

Means designed by the same letter are not significantly different at the 5% level according to Duncan's test.

The favorable effect of organic treatments on vegetative growth, especially at the early stage of plant growth may be due to that compost made from biosolids contains almost all of the macro- and micro-nutrients essential for plant growth (Table 2), in addition to humic substances which increased soil fertility and cation exchange capacity, thus increased the availability of certain nutrients (Seyedbagheri 1999). Also, applying compost improved physical conditions of soil, providing energy necessary for microorganisms activity and increasing the availability and uptake of nutrients, which positively reflected on vegetative growth (Awad 1998, Romero et al. 2000, Bayoumi 2005; Ehaliotis et al. 2005). The stimulation of plant growth by using compost + compost tea or seaweed extracts may be attributed to the combined effect of compost, compost tea (which contains humic acids, vitamins, amino acids and both of macro and micro nutrients which enhanced cucumber growth) and seaweed extracts which contains some growth regulators such as cytokinins (Brain et al. 1973), auxin (Temple and Bomke 1989) and gibberellins (Williams et al. 1981).

3- Chlorophyll content

Data in Table 3 indicate that, chlorophyll content (SPAD green colour reading) was highly significant influenced by different organic and mineral treatments at the two sampling dates (45 and 60 days after transplanting) in both seasons. At the first date, both of Tr. No. 2 (compost + compost tea + 0.05 mM of BTH) and Tr. No. 3 (compost + seaweed extract + 0.05 mM of BTH) showed the highest values of chlorophyll content in leaves in both seasons. In contrast, the lowest values were obtained from applying Tr. No. 5 (mineral fertilizers alone) which sometimes don't differed with Tr. No. 4 (Mineral fertilizers with 0.05 mM of BTH).

At 60 days after transplanting, the highest values were obtained from Tr. No. 4 (Mineral fertilizers with 0.05 mM of BTH) and Tr. No. 3 (compost + seaweed extract + 0.05 mM of BTH), but the lowest values were showed from Tr. No. 1 (compost + 0.05 mM of BTH) and Tr. No. 5 (mineral fertilizers alone) in most cases.

The superiority of organic treatments (No. 2 & 3) in chlorophyll content at the early stage may be due to the higher

Table 5. Effect of organic and mineral treatments on chemical quality of cucumber fruits in 2005 and 2006 seasons.

Treatments	Ascorbic acid content (mg/100 g fresh wt.)	Total soluble solids (%)	Nitrate content (ppm)	Ascorbic acid content (mg/100 g fresh wt.)	Total soluble solids (%)	Nitrate content (ppm)
	2005 season			2006 season		
1. Compost + BTH	13.9 b	4.3	110.2 b	15.2 b	4.2	95.6 b
2. Comp.+ Comp. tea + BTH	14.7 a	4.5	110.5 b	16.2 a	4.4	96.2 b
3. Comp. + Seaweed ext. + BTH	14.1 ab	4.6	112.0 b	15.9 a	4.3	105.4 b
4. Mineral fertilizers + BTH	12.1 c	4.9	253.1 a	14.6 b	4.7	233.4 a
5. Mineral fertilizers alone (control	11.6 c	4.8	236.4 a	13.3 c	4.6	220.9 a
F-test	**	N.S	**	**	N.S	**

Means designed by the same letter are not significantly different at the 5% level according to Duncan's test

biological activity of soil which encouraged the availability of nutrients and produced high energy helping in root development (Li et al. 2000). Also, compost, compost tea and seaweed extracts contain considerable amounts of macro- and micro-nutrients, amino acids, vitamins and hormones as mentioned before which possibly increased chlorophyll content leading to higher rates of photosynthesis. In this concern, Mengel and Kirkby (1987) stated that Mn and Fe play an important role of porphyrine structure of chlorophyll. On the other hand, the favorable effect of chemical treatment on chlorophyll of cucumber leaves at the late date may be due to more availability of inorganic N form for uptake by plants.

4-Fruit yield

It is obvious from Table 4 that, significant differences in early and total yields (number and weight of fruits/m^2) were noticed among the used five treatments in both seasons. Using both of Tr. No. 2 and 3 resulted in an increase in early yield (number and weight of fruits/m^2) in most cases compared to chemical treatments especially without 0.05 mM of BTH, which produced the lowest early yield in both seasons.

Applying chemical fertilizers with 0.05 mM of BTH (Tr. No. 4) produced the highest number and weight of fruits/m^2 of total yield. On contrast, using organic treatment No. 1 (compost combined with 0.05 mM of BTH) showed the lowest total fruit yield in both seasons.

The performed higher early yield from organic treatments (No. 2 & 3) than chemical treatments (No. 4 & 5) may be due to their higher nutritional contents, particularly Fe, Zn and Mn in compost (Table 1) and K, Ca, Mg, S and Fe in seaweed extract. These elements can encourage the vegetative growth and total chlorophyll (Table 3) and the photosynthetic rate which enhance flowering and fruiting leading to an increase in early fruit maturity.

These results agree with those of Hsieh and Hsu (1995), Abd-Allah et al. (2001), Aly (2002), Bayoumi (2005) and Ehaliotis et al. (2005), they showed that applying of organic treatments increased early yields compared to using chemical fertilizers. Also, Rotenberg et al. (2005) reported that Additions of organic amendments (composts) to agricultural soils can lead to improved soil quality and reduced severity of crop diseases as well as increased cucumber yield.

The greater cucumber yield with chemical treatment with 0.05 mM of BTH (No. 4) may be a result from increased N concentration or accumulation as suggested by Sainju et al. (2001). Also, the present study indicate that organic Tr. No. 3 can produce higher fruit yield comparable with those of chemical treatment. These results agreed with those of Montagu and Goh (1990) and Poudel et al. (2002).

5- Fruit quality

Data presented in Table (5) indicate that both of ascorbic acid and nitrate contents were significantly affected by treatments. Therefore, any of either Tr. No. 2 or Tr. No. 3 showed the highest content of ascorbic cid followed by Tr. No. 1, while using chemical Tr. No. 4 or No. 5 led to give the least values. On the other hand, chemical Tr. produced fruits having the highest nitrate content compared to organic Tr. No., 1,2 and 3 which produced fruit having the lowest nitrate values. Similar conclusions were drawn by Yacheva et al. (1982), Vogtmann et al. (1993), Abou-Hussein (2001) and Poudel et al. (2002).

The highest nitrate content due to chemical fertilizers may be attributed to that mineral fertilizer salts are soluble and nitrogen is immediately available for plant uptake soon after fertilizer application. Otherwise, organic N fertilizers release nutrients slowly (Haworth 1961).

For TSS %, data show that, it was non-significantly influenced by treatments. However, chemical treatments (4 &5) tended to produce higher values of TSS than the other organic treatments (1,2 &3) in both seasons.

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DISSERTATION SUMMARY

Biohydrogen production from keratin-containing animal wastes

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Meat processing industry produces many kinds of keratinous waste materials (animal feather, wool, hair), which are degraded very slowly in nature, therefore, it is regarded as hazardous waste according EU directives. Decomposition methods like incineration or chemical treatments are employed (Onifade et al. 1998), although these procedures are rather expensive or environment-polluting. In contrast, biotechnology offers environmentally sound and cheap or even economical biodegrading methods. Recently, a two stage fermentation system was constructed to convert keratin-containing biowaste into a useful product, biohydrogen (Balint et al. 2005). A keratin-degrading Bacillus strain was isolated and tested in waste decomposition experiments (Perei et al. 2000). During the biodegradation process keratin-containing material was converted into a fermentation product which was rich in amino acids and peptides. This mixture could be subsequently used as major nutrient source for an anaerobic hyperthermophilic achaeon, Thermococcus litoralis, which produced hydrogen gas as a physiological byproduct.

The conceptual setup of the two-stage fermentation system

Several keratinaceous wastes (chicken feather meal, goose feather meal, pig hair) were digested by Bacillus licheniformis KK1 and the fermentation product - supplemented with essential minerals - was subsequently used as nutrient for T. litoralis. Cell propagation, nutrient uptake and biohydrogen production were followed. The archaeon was found to utilize all three fermentation broths for biohydrogen production similarly to bacto-peptone, the standard peptidic growth substrate for T. litoralis in medium 623 (DSMZ). Besides T. litoralis two microbes (the gram negative E coli and the gram positive Caldicellusiruptor saccharolyticus) capable to produce hydrogen were examined but neither of them could utilize the keratin hydrolysate for biohydrogen production.

Optimization of the keratin-degradation step

Feather meal degradation was subsequently optimised for the hydrogen production step and the optimal feather digestion time was investigated in few pH controlled experiments performed in a 700 ml batch fermenter. Protein concentration of the cell-free fermentation broth was followed during fermentation and was found to increase significantly; 64% of the applied feather meal was solubilised within 60 hours. The picture of the samples taken from the fermenter and analysed on polyacrylamide gel confirmed that the feather materials were converted into small-sized soluble peptide fragments.

Scale-up of the hydrogen producing step

Hydrogen production studies were performed in a 7 l fermenter at 85°C, pH=6,5, under nitrogen. Cell growth, consumption of alkali (used to maintain pH), nutrient uptake and biohydrogen production was monitored. All the followed parameters indicated substantial metabolic activity of the hydrogen producing cells in the first 30 hours of fermentation. During this period 50% of the available peptides in the fermentation broth was utilized. Hydrogen production yield was found significantly higher than the earlier yields of small-volume pilot experiments.

Conclusion

Work presented here demonstrates the feasibility of biohydrogen generation from keratin-rich biowaste. Using the developed two-stage fermentation system it is possible to utilize animal feather, wool, hair and other protein-rich biowastes for biological hydrogen production.

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DISSERTATION SUMMARY

Changes of chondrocyte gene expression under inflammatory conditions

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Arthritis is a degenerative arthropathy that frequently leads to chronic pain and disability. With the aging of our population, this condition is becoming more prevalent and its treatment increasingly financially burdensome. Finding better treatments for arthritis is a major focus of medically oriented research. This disease is characterized by joint swelling and degradation of the articular cartilage matrix. If we could understand and repress these processes we will be able to give back not only the freedom of moving, but to repress the amount of the analgesic drugs.

Our aims were to map in chondrocytes the inflammatory signal transduction pathways, to compare that with another cell's analogous pathways, to determine possible targets of antirheumatic drugs. To accomplish our goals we needed a test system, which can reflect the behavior of the chondrocytes during (in vivo) inflammation, and will be sufficient to test anti-inflammatory drugs.

The commercially available SW1353 human chondrosarcoma cell line and a rat chondrosarcoma cell line (RCS, Mukhopadhyay et al. 1995) were included in our studies.

In the SW1353 human chondrosarcoma cell line gene expression has been studied by RNA-blot hybridization and RT-PCR. We used GAPD and rRNA as standards. Proinflammatory cytokines induced characteristic changes in the gene expression pattern. After 24 hr induction with interleukin-1 (IL-1) elevated level of MMP13 and MT1-MMP mRNAs was seen. These enzymes are responsible in large part for the degradation of extracellular matrix in arthritic cartilage. At the same time significant decrease of the RNAs encoding the chondrogenic master transcription factor Sox9, and the cartilage specific matrix molecule collagen type II was also observed. Transient increase of the Egr1 mRNA level was also monitored. RCS cells responded well to the inflammatory induction, too.

We followed the nuclear factor κB (NFkB) mediated changes by using SW1353 clones stably transfected with plasmids encoding NFkB-driven luciferase marker gene. We confirmed with the behavior of the transfectants, that the inflammatory symptoms inducible with IL-1, tumor necrosis factor α (TNFα), phytohemagglutinin (PHA), and phorbol 12-myristate 13-acetate (PMA) were mediated at least partly by NFkB.

Several anti-inflammatory drugs were tested in the model cell culture system. PDTC was effective only at high concentrations detrimental to the cells. In some other cases the compounds were effective only at special conditions. Some of the observed discrepancies can be attributed to the difference of the cell line, culture conditions and the proinflammatory agents used in our laboratory and published in the literature. Diacerhein and CTP-N-TPC (Sullivan et al. 1998) in our hands were able to inhibit the effect of PMA and PHA in serum free conditions, but had no effect in case of the induction with IL-1 in SW1353 clones, in contrast to the RCS clones where diacerhein reduced the effect of IL-1 in the presence of serum.

We produced primary cultures of articular chondrocytes and synovial fibroblasts to compare those with the permanent cell lines.

In conclusion the proinflammatory drugs we tested on these two cell lines were able to induce the inflammatory pathways, the luciferase assay supported that NFkB-mediated signal transduction takes part in this induction, and we could make appropriate circumstances in which the anti-inflammatory drugs are effective and are useable to further tests.

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DISSERTATION SUMMARY

The functional investigation of the interaction between p53 and *Drosophila melanogaster* TAF(II)155/Bip2 and the human homolog, TAF3

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The tumor suppressor gene *p53* plays a pivotal role in safeguarding the integrity of the genome (Levine. 1997). Most human tumors have a mutation in the *p53* gene or a functional defect in the p53 pathway, highlighting its importance for preventing tumorigenesis. p53 is a sequence-specific transcription factor. Normally, the amount of p53 protein in a cell is kept at a low level. Cellular stresses, such as DNA damage, hypoxia, or abnormal oncogene activation, signal to p53, stabilize and activates it as a transcription factor, p53 in turn arrests cell cycle, induces repair and apoptosis. The mechanisms activating p53 are not fully characterize therefore we were interested in identifying novel proteins that regulate p53. The discovery of *Drosophila melanogaster* p53 (Dmp53) facilitated the examination of p53.

Using yeast-two-hybrid screen several new interacting partners of Dmp53 were identified. One of the identified genes- TAF(II)155/Bip2- was chosen for further analysis. Bip2 is a novel *Drosophila* TATA-box Protein Associated Factor (TAFII), is also named TAF(II)155/Bip2 and its human homologue is a TAF3 (Pointud et al. 2001). The TFIID, RNA polimerase II transcription factor is composed of TATA-binding protein (TBP) and TBP-associated factors (TAFs). The TAF(II)155/Bip2 and its human homologue TAF3 protein contain a Histone Fold Domain (HFD) in the N-terminal region and a Plant Homeodomain (PHD) in the C-terminal region (Gangloff et al. 2001).

Because of the results received in yeast-two hybrid experiment we examined the possible interaction between the human homologue of these *Drosophila* proteins. Surprisingly, the *Drosophila* homologue (Bip2) can also interact hp53 and with the family members of hp53 (hp73α and β) not only with Dmp53 in a yeast-two hybrid assay. First we amplified the hTAF3 from HeLa cDNA library, but we could not amplify the full length form of hTAF3, only two partial length forms. In our experiments we used this longer and shorter forms. To examine if there is any effect of the overexpression of the human homologue (TAF3) on the transactivation activity of hp53, and the members of hp53 family (p73α, p73β), we transfected HeLa cells with the hp53 and TAF3 overexpressing constructions and a luciferase reporter plasmid, we found that the overexpressing of TAF3 decreased the transactivation activity of hp53 and p73β and a lesser extent in the case of p73α. Using RNA interference (RNAi), we investigated the effect of the elimination of TAF3 protein on the transactivation activity of hp53. We created double-stranded RNAs to the TAF3 and we cotransfected it with hp53 expressing plasmid and a luciferase reporter plasmid into HeLa cells. In some cases the dsRNA-TAF3 was able to decrease the transcriptional activity of hp53, but in other cases we could not detect this result. To examine the subcellular localization of TAF3 we transfected HeLa cells with TAF3 overexpressing vectors and we stained the cells with primary and fluorescein-conjugated secondary antibody, we found that the hTAF3 protein showed really nuclear localization in HeLa cells. To further prove the physical interaction in vitro between the interacting partners and Dmp53, we used GST-pull down assay, we found that the TAF(II)155/Bip2 is able to bind to the Dmp53. The other method that we used is the Immunoprecipitation (IP), but by this technique we could not detect interaction between hp53 and the two different size of TAF3. On the other hand it is possible that the TAF3 cDNA clone in our hand does not contain the region recquired for interaction. Therefore we will use the full length mouse homologue of TAF3 (TAFII140), which was amplified and offered for us by our cooperating partner.

We plan to investigate the interaction between hp53 and mTAF3 (TAFII140) using of GST-pull down and IP experiments.

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DISSERTATION SUMMARY

A toxin-antitoxin module in *Sinorhizobium meliloti*

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The chromosomal *ntrPR* operon of *Sinorhizobium meliloti* encodes a protein pair, which forms a toxin-antitoxin (TA) module; the first characterized functional TA system in *Rhizobiaceae*. A typical TA module consists of two small genes that form an operon, in which the first gene determines an unstable antitoxin and the second gene, a stable toxin protein. The toxins and antitoxins of different TA pairs may belong to unrelated superfamilies. Seven TA gene families were described (Gerdes et. al. 2005); one of them is the most abundant *vapBC* family present in Gram-positive and Gram-negative bacteria as well as in Archaea. The operon organization and domain architecture of the TA modules from this family resemble those of the NtrPR proteins: the antitoxin is an AbrB/MazE homolog and the toxin belongs to the PIN domain family.

The first TA modules were identified on plasmids acting as post-segregational killing systems. Their function was to prevent the proliferation of plasmid-free progeny. In further experiments TA loci were also identified on chromosomes and were considered to be associated with the modulation of the global level of translation under conditions of nutrient limitation, or under various stress conditions.

We have shown that the autoregulatory functions of *ntrPR* operon are in accordance with other TA systems: the antitoxin NtrP is able to recognize a DNA segment in the promoter region of the *ntrPR* operon, but its binding is weak, resulting in an unstable DNA-protein complex. The toxin component alone is not able to bind to the same DNA region, but the complex of NtrP and NtrR strongly binds to the promoter region resulting in the negative autoregulation. The N-terminal part of NtrP is responsible for the interaction with the promoter DNA, whereas the C-terminal part is required for protein-protein interactions. The NtrR protein plays a role in the stabilization of the complex, but it has no promoter binding activity.

We determined that the binding site of the NtrPR complex is a direct repeat sequence that partially overlaps the transcription site.

Experiments focusing on the possible function of this operon revealed that a Tn5 insertion in the ntrR gene resulted in increased transcription of *nod* and *nif* genes as compared to that of the wild type strain, and this effect was more pronounced in the presence of an external ammonium source (Dusha et al. 1989; Oláh et al. 2001). When the gene expression patterns of the entire genomes of the wild type and *ntrR* mutant strains were compared under oxic and microoxic conditions, an unexpectedly large number of genes exhibited altered expression in the mutant strain (Puskás et al. 2004).

In order to examine a possible toxic function of NtrR, we tested the growth and viability of *E. coli* derivatives carrying plasmids with *ntrR*, *ntrP*, or both genes controlled by the arabinose inducible promoter. NtrR overexpression resulted in the inhibition of cell growth and colony formation, but this effect was counteracted by the presence of the antitoxin NtrP.

These results and our earlier observations demonstrating a less effective down-regulation of a wide range of symbiotic and metabolic functions in the *ntrR* mutant under microoxic conditions and an increased symbiotic efficiency with the host plant alfalfa suggest that the *ntrPR* module contributes to adjusting metabolic levels under symbiosis and other stressful conditions (Bodogai et al. 2006)

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DISSERTATION SUMMARY

Usage of enumeration method based algorithms for finding promoter motifs in plant genomes

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Understanding the underlying genetic and physiological processes would make it possible to enhance crop yield in the face of abiotic stress. To do so would entail unraveling the complex regulatory interactions behind this phenomenon. The analysis of transcription factor binding sites in promoters is a common and useful tool in such studies.

Our work focused on developing a number of computer algorithms for finding motif dyads in a set of input promoter sequences for co-regulated genes, since they tend to group together into regulatory complexes in promoters. Our algorithms belong to the family of algorithms called enumeration methods, which perform an exhaustive search for all possible motifs in the input sequences. In our case we looked for dyad motifs, described by the formula $M_H N_n M_T$, where M_H and M_T are individual motifs, each the same length, and N_n represents a spacer between the head and tail motifs n bp long.

For each algorithm we counted the number of head and tail motifs as a function of spacer length. The basic logic behind each algorithm was to quantify the difference between the distance distribution of a given dyad and the uniform distribution. We assumed that if a given dyad is biologically irrelevant, then its head and tail motifs would occur randomly at all distances from each other in equal numbers, thereby producing a nearly uniform distribution. If the dyad has a biological function, then they should occur at a given distance from each other in high numbers. In this case, the distribution of the head and tail motifs should depart from the uniform distribution, and accumulate at a specific distance from each other.

The first and second algorithm measures the difference between the maximal occurrence and the average occurrence of the dyad according to motif distance. Greater differences infer biological relevance (Cserháti 2005). The third algorithm represents individual motif distances with "boxes" into which a number of "balls" are distributed, which represent the occurrences of the dyad at that motif distance. The algorithm calculates the probability of this distribution. Less probable distributions can be assumed to be more relevant. The fourth algorithm calculates the level of homogeneity between the distribution of the dyad in the input promoter set and a set of randomly selected promoters (Cserháti 2006).

We tested our four algorithms on 130 *Arabidopsis* stress genes, and searched for pentamer dyads with a maximum distance of 52 bp in between. The top 50 dyads were selected for each method, and the *Arabidopsis* promoterome was screened to find promoters with high motif content. 72.3% of the original promoters could be found back with our methods, and 58.7% were found to be involved in stress. The fourth method has already been experimentally verified by comparing the number of dyads in the family of rice aldo-keto reductase genes.

Another work involved reviewing 151 CDK genes in plants. Overall, 29 genes were found in *Arabidopsis*, and 30 in rice. Promoter analysis was performed for 26 of these genes. More motifs were found in rice, which were involved in ABA and auxin response. Ethylene response elements were also found in CDKB's and CDKC's, but not in CDKA's. A number of elements were found which are involved in light response and Circadian rhythm. The MSA, MYB, and APETALA3/AGAMOUS motifs were also found in a large number of promoters.

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DISSERTATION SUMMARY

Isolation, cloning and characterization of satellite DNA families in rabbit

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The pericentromeric heterochromatin of mammalian chromosomes is composed of tandemly repeated satellite DNAs, frequently forming arrays of several million nucleotides. Satellite compositions of centromere regions are variable, indicating that it is epigenetic modification, rather than the particular sequence of nucleotides, what supports centromere function. The rabbit satellites described below are arrays of medium-sized monomer units with family-specific features. They have not only different lengths (~375 and ~585 bp), but completely different sequences, as well. Individual Rsat I sequences showed differences in the 5.3–21.8% range (78.2–94.7% identities), and that of Rsat II/IIE was 8.0 – 22.2% (77.8–92%). The rabbit satellites appear to be more homogenous than their human counterparts, owing to the less extensive higher-order repeat organization. FISH experiments with Rsat I, Rsat II and Rsat IIE showed, that the satellite sequences identified so far did not cover the complete chromosome complement of rabbit, although signals were detected with Rsat I on 11, with Rsat II to 12, and with Rsat IIE to 2 chromosome pairs. It is remarkable that both Rsat I and Rsat II were found on nine chromosomes, but Rsat I was undetectable on the two chromosome pairs (#9 and #16) with Rsat IIE. Apparently, the two main satellite families have always been, or have become so different that they could coexist without much influence on each other. Probably, these two sequences are equally compatible with centromere function. The fact that the Rsat IIE-bearing chromosomes still carry Rsat II supports the view that probably the former arose from the local amplification of the latter. Processes in accordance with the „library" hypothesis of satellite DNA sequence evolution appear to be at work in rabbit in two forms. First, both of the two main families, Rsat I and Rsat II, were detected together in varying ratios on a number of chromosomes, forming thus major components of the library. Second, the emergence of Rsat IIE on chromosomes #9 and #16 is the result of a relatively recent branching from Rsat II, thus creating new „volumes". However, the catalogue of the rabbit satellite „library" is still incomplete, since there are still nine chromosomes (seven somatic pairs and the sex chromosomes) for which no satellite has been characterized so far. The possibility that these chromosomes had some variant of either Rsat I or Rsat II seems unlikely, since no FISH signal was detected with any of these probes under low-stringency conditions. Although both the Rsat II and Rsat IIE arrays are based on similar, but diverged repeat units, only Rsat II showed signs of higher-order periodicity. Therefore, it is plausible that the Rsat IIE arrays of these chromosomes formed via relatively recent large-scale amplification events of diverged repeat units, and consequently, they are expected to be more homogenous. Since the Southern hybridization pattern of Rsat IIE is more regular than that of Rsat II, such putative saltatory amplification events could indeed have occurred. Taken together, it is plausible that Rsat IIE was derived from divergent Rsat II units, and not vice versa. The use of an rDNA hybridization probe showed that in addition to the three known NOR chromosomes, there were signals at 21q(ter). This locus is as a useful cytological marker at least in some species of the Leporidae. While attempts are made to identify the missing satellites, those present on the NOR chromosomes could become components of rabbit satellite-based artificial chromosomes.

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DISSERTATION SUMMARY

Examination of small antimicrobial proteins and their genetic determinants

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The number of fungal infections has increased continuously over the past years. Infections caused by opportunistic filamentous fungi are especially problematic, because most of the antifungal treatments available have serious side effects and could not be applied without a damage of the host (Vicente et al. 2003). Therefore, there is a substantial demand for new types of compounds with antifugal activity. The defensin-like proteins secreted by some filamentous fungi are interesting from this respect, as they have effective inhibitory potencial both on the hyphal extension and on the germination of the spores. Colllective characteristics of these proteins are the low molecular mass (5.8-6.6 kDa), basic character, 6-8 cysteine residues, and the presence of several disulfide-bonds. Similar proteins have been found and investigated from five fungal species (*Penicillium chrysogenum*, *P. nalgiovense*, *Aspergillus giganteus*, *A. niger*, *Gibberella zeae*); among them only the AFP (*A. giganteus* antifungal protein) from *A. giganteus* and the PAF (*P. chrysogenum* antifungal protein) by *P. chrysogenum* are studied intensively. They have a narrow antimicrobial spectrum, but their specificity are different (Marx 2004). It has been proved that PAF is very effective against opportunistic patogenic zygomycetes (Galgóczy et al. 2005), at the same time it does not harms human cells (e.g. immune cells, nerve cell) *in vitro*. The GAMA (*G. zeae* antimicrobial protein) from *G. zeae* (teleomorf of *Fusarium graminearum*) is a hypothetical protein derived from genomic DNA sequence database. Based on these data, experiments have been carried out to identify new antifungal proteins and their genetic determinants in the genus *Fusarium*.

Fifteen isolates, representing 10 *Fusarium* species (*F. graminearum*, *F. asiaticum*, *F. boothi*, *F. cerealis*, *F. culmorum*, *F. avenaceum*, *F. poae*, *F. polyphialidcium*, *F. sporotrichioides* and *F. pseudograminearum*) have been screened via PCR experiments. Sequences corresponding to hypothetical defensin-like proteins have been found in all isolates. These revealed high similarity to the nucleic acid sequence of the paf gene. Taking into account their nucleic acid and hypothetical protein sequences, 4 types of *Fusarium* antifungal proteins could be differentiated.

The production of antifungal poteins have been optimized. Their biological activities on hyphal growth of *Trichoderma longibrachiatum* and *Mortierella elongata* with an agar diffusion technique have been investigated. Eight of ten showed similar inhibitory effect (like PAF), while the ferment broth of two species (*F. sporotrichioides* and *F. asiaticum*) proved to be inactive in these tests. The supernatant of *F. polyphialidicum* was the most effective in the inhibition of the hyphal extension.

Protein gel electrophoresis revealed the presence of a small protein (approximatly 6.3 kDa) in the eight biological active species. These proteins have been purified further (e.g ultrafiltration). The partially purified protein of *F. polyphilaidicum* maintained its antimicrobial activity. It was supposed, that this 6.3 kDa protein responsible for the antifungal activity, and it was named *Fusarium poliphyalidicum* antifungal protein (FPAP).

The effect of FPAP on germination efficiency of sensitive conidiospores was examined in *T. longibrachiatum*. The conidiospores displayed abnormal, and delayed germination when cultivated in a FPAP-containing medium compared to a control. FPAP-treated conidiospores formed very short, swelled hyphae with multiple branches.

FPAP is a new, small antifungal protein. Further experiments are in progress to clarify its antifungal spectrum, and its effect on plant and mammalian cells.

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DISSERTATION SUMMARY

Physiological studies on hydrogen-evolving and diesel-degrading microorganisms

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The physiology of hydrogen-evolving anaerobic bacteria has been studied and their physiology improved by use of additives (Andersson et al. 2000; Halverson et al. 2000). The use of 3% polyethyleneglycol 8000 added to the growth media of *Enterobacter cloaceae* improved the viability and increased the hydrogenase activity 2-fold compared to cells grown in additive-free growth medium. It also exerted a positive effect over the strict anaerobic extremophiles *Termococcus litoralis* (1.4-fold increase in hydrogenase activity), *Fervidobacterium pennivorans* (1.7-fold increase) and *Caldicellulosiruptor saccharolyticus* (1.7-1.9-fold increase). The use of the biodegradable polymer Na-alginate (Ertesvag H and Valla S 1998; Vancov T et al. 2005) has had even higher effect - 4-fold increase of hydrogenase activity for *E. cloaceae*, 2.2-fold for *T.litoralis* and 3.1-3.3-fold for *C. saccharolyticus*. The additives have also had a favourable effect over the cell physiology when immobilized onto solid support matrices (perlite, granulated active carbon, wooden chips) and stored semi-aerobically at room temperature. Additive-treated immobilized stored cells showed a better recoverability and a prolonged activity over time.

A study on soil microbial physiology upon diesel perturbation has been conducted. The dilution method (Griffiths et al. 2001) was implemented. The incubated soil has been contaminated with 2% (v/w) diesel fuel. The diesel degradation rates and the metabolic activity (Biolog GN2) of the soil microbes have been determined. Qualitative and quantitative analysis of diesel - contaminated and non-contaminated microbial and fungal soil communities has been performed on Beckman CEQ 8000 genetic analysis system, using T-RFLP technique based on the 23S bacterial ribosomal DNA and ITS2 fungal ribosomal DNA).

A total of 71 different species were detected. The dilution technique for amending diversity was particularly effective with regard to the fungal component of the community. Diesel-treated soils developed fungi from the genera *Mucor, Geotrichum, Rhitophthora* and *Sporobolomyces*, while non-diesel soils were prevailed by *Gliocladium* and *Verticullum* genera. Diesel amendment reduced diversity (in both dilution treatments) and several bacterial species (*Mycobacterium spp., Rhodobacter spp., Burkolderia spp.*) were only detected in diesel-contaminated soil. Community level catabolic potential was generally unaffected by diesel contamination when all 95 substrates were considered. Oxidation of the simple carbon substrates (e.g. carbohydrates) was unaffected by diesel, but utilisation of more complex substrates (e.g. aromatic compounds) was increased when communities were subjected to diesel contamination. This suggests redundancy of easily degraded substrates and a diesel-mediated selection mechanism leading to more complex degradation capabilities in communities associated with diesel-contaminated soils. Diesel degradation was more rapid in soils with the greatest species-richness than in those with the lower levels of diversity. This was the case for every hydrocarbon measured (C_{12} to C_{25}).

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DISSERTATION SUMMARY

Advances in gene expression based molecular diagnosis

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Global transcription profiling with DNA microarray technology has lead to a deeper understanding of the sophisticated cellular processes. Pathological alteration, as a complex biological process, is constantly being studied in this manner in a quest to find key drug targets. However, the large data sets comprising simultaneous expression levels of thousands of genes monitored under diverse circumstances still constitute great challenge for biologists as well as computational algorithm developers. It is known that various treatment procedures may have different effects on patients diagnosed as having the same type of cancer due to different origins or courses in the development of the tumor. Although patients suffering from leukemia may have similar symptoms, it has been shown that microarray generated gene expression patterns are capable of making the distinction between the different subtypes of the disease (Golub et al. 1999). Over the last few years many molecular classification approaches based on statistics or machine learning algorithms have been applied to microarray data. Their common feature is that they try to model classes of *a priori* annotated samples by means of supervised training. With the obtained model parameters they predict the belonging of an un-annotated sample to one of the known classes. So far the support vector machine (SVM) has been shown to have the best performance for microarray classification problems. It has been successfully applied with a variety of binary and mutli-class tumor classifications (Ramaswamy et al. 2001). Notable performance was also obtained with artificial neural networks (ANN) (Khan et al. 2001).

Here we propose the use of the linear Kalman filter (Kalman 1960) as a preprocessing step in microarray based molecular diagnosis. Taking into account the expression covariance between genes is desired in such classification problems, since this stands for the functional relationships that govern tissue state. Hereby, we show that employing the Kalman state estimator to remove functional noise yields linearly separable data, suitable for most classification algorithms.

It is known that microarray data are usually corupted with measurement noise from various sources. Some percentage of the variance of a measured gene-expression signal is also due to biological variation. We sought to use the Kalman filter to remove measurement and functional noise, modeled as normally distribuited random variable and to estimate the biological state. Therefore we built a simple measurement state-space model:

$$x_i = x_{i-1} + w_i$$
$$y_i = x_i + v_i$$

where y_i is a numerical vector containing the expression values measured from the ith sample, x_i is the filtered expression data and thus the biological state, and v_i and w_i are noise and biological, allowed variation respectively. To reduce dimensionality, we applied singular value decomposition (Alter et al. 2000). We then employed Kalman filtering on the obtained model with tuning beeing done solely on the training dataset.

We applied the method on publicly available datasets and we found that it boosts the performance of the widely used ANN, SVM, k-nearest neighbours and classification trees, improving diagnosis accuracy. Kalman filtering also greatly improves the graphical viasualization of microarray data.

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DISSERTATION SUMMARY

Characterisation of anticryptococcal Fc-1 toxin of *Filobasidium capsuligenum*

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The basidiomycetous yeast *Filobasidium capsuligenum* (IFM 40078) produces a killer toxin (FC-1) which is highly effective against the opportunistic fungal pathogen *Cryptococcus neoformans*. The sensitivities of strains representing eight molecular subtypes (VNI-IV and VGI-IV) of the *C. neoformans* species complex, and of an additional 50 clinical and environmental isolates were determined. Additionally further yeast species were tested for toxin sensitivity, but FC-1 showed strong specificity for *Cryptococcus* isolates, and did not affect the other examined yeast species. This highly specific effect is mainly characteristic to proteins. Several other observations such as the high thermolability of FC-1, the partial loss of activity in presence of proteases and the considerably narrow pH optimum (pH 4-6) also confirm the protein character of the toxin. Our goal was to characterise and clone the toxin encoding gene and to study the effect of the toxin on *C. neoformans* cells.

Neither RNA, nor DNA plasmids were detectable by agarose gel electrophoresis or pulse-field gel electrophoresis (OFAGE), thus the gene encoding the toxin is most likely located in the chromosomal DNA.

Growing of *Filobasidium capsuligenum* (IFM40078) on complete media leads to toxin production, however on minimal media no biological activity can be detected. In order to identify the toxin the extracellular enzyme profiles of the fungi were determined by polyacrylamide gel electrophoresis in both conditions. Comparison of the results showed that there are a large number of differences between the two profiles making it difficult to unambiguously identify the toxin. Similar observations were perceived when profiles of non-producing *Filobasidium capsuligenum* strain (VKM1513) or non-producing mutant strains generated by UV mutagenesis were compared to the toxin containing profile of strain IFM40078.

Results of competition assay suggest that β-1,6-glucan (pustulan) in the cell wall may provide the binding site for the killer protein. Taking advantage of this specific binding β-1,6-glucan was applied for affinity chromatography as a ligand to purify the toxin. Biologically active fractions from the pustulan-coupled epoxy-activated sepharose 6B column were loaded on polyacrylamid gel. A few proteins in the range of 19 - 51 kDa were detected. A 47 kDa protein, the best candidate for being the toxin, was sequenced (XVNVNGVPI). This sequence could be used for aquiring the coding DNA by reverse transcription.

Other, already characterised killer toxins have been used for homology searches to find conservative amino acid or nucleic acid regions. However no significant homology has been found among these proteins suggesting that such comparative studies do not serve as useful tools for further characterisation of the toxin.

An effective approach for identifying the coding DNA can be the analysis of the toxin producing (IFM40078) and non-producing (VKM1513) strains at the mRNA level. By subtractive hybridisation cDNA segments differing in the two strains can be selectively amplified in order to find the toxin encodig gene.

To investigate the mechanism of the effect of the FC-1 toxin-treated cells were studied. Analysis of cellular DNA by laser scanning cytometry and FITC staining revealed that the killing mechanism of FC-1 is neither cell cycle- nor cell wall biosynthesis-dependent; rather it might act as an ionophor that disrupts the cytoplasmic membrane function.

These various research tools could lead to the complete characterisation of the FC-1 anti-cryptococcal toxin which has the potential to be applied as a therapeutic agent for the treatment of cryptococcosis.

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DISSERTATION SUMMARY

Protein electron transfer. Spectroscopy and modeling

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Redox (electron transfer) reactions are widespread in living organisms and are of utmost importance in biological energy conversion. Most life forms depend on either photosynthetic or respiratory ATP production. Both of these are intimately linked to long range intra- and interprotein electron transfer processes coupled to transmembrane proton transport, which results in the energized state of the membranes hosting the ATP synthase enzyme. Electron transfer in proteins takes place between redox cofactors separated by distances well above 10 Å in some cases and yet, the electron transfer is sufficiently rapid for efficient energy conversion (usually faster than milliseconds).

Marcus theory describes the rate of electron transfer in the non-adiabatic, outer shell case applicable to redox proteins, with parameters characteristic of the physical properties of the medium. These parameters can be viewed in two different ways (Jones et al. 2005)

One intepretation consideres the protein as a semi-homogeneous and semi-isotropic medium whose actual structure can be characterized solely by its packing density as far as electron transfer efficiency is concerned. In the other interpretation the chemical composition and secondary and tertiary structural elements are explicitely taken into account in calculating the optimal electron transfer pathway connecting donor and acceptor.

We are experimentally studying the electron transfer rate by kinetic spectroscopy, using the photoactive covalent redox label TUPS (thiouredopyrene-trisulfonate; Kotlyar et al. 1997). Two forms of TUPS can bind to either lysine or cysteine side chains. Photoexcitation of the label by a 355 nm laser pulse (Nd-YAG third harmonic) yields the triplet excited state of TUPS, which is a good electron donor or acceptor – depending on the redox partner (Kotlyar et al. 2004). We have attached TUPS to genetically engineered cysteine side chains of horse heart cytochrome c. In collaboration we have also measured electron transfer on TUPS labeled flavodoxin, as well as in the complex of TUPS labeled cytochrome and cytochrome c oxidase.

We have mapped the entire surface of horse heart cytochrome c in terms of electron transfer coupling leading to the heme redox cofactor using available pdb structures and the program HARLEM (www.kurnikov.org). In this way we can identify potential "cold" and "hot" regions in the protein for electron transfer, and assist future site directed mutagenesis for positioning the redox label at locations of particular interest.

Both on cytochrome and on flavodoxin the kinetics of electron transfer between TUPS and the respective redox cofactors (heme and flavin) are more complex than expected. We have performed molecular dynamics calculations to demonstrate that TUPS can occupy several equilibrium positions relative to the protein surface. Electron transfer pathway (and packing density) calculations have been carried out for these conformations. It appears that the optimal electron transfer pathways do not necessarily follow the covalent link between TUPS and the redox cofactors, but may involve through space jumps from the label to the protein surface. The correlation between the measured electron transfer rates and the pathways so calculated turns out to be much better than the correlation between the rates and the covalent distance between donor and acceptor (Tenger et al. 2005).

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DISSERTATION SUMMARY

The effect of chronic alcohol consumption on nitric oxide synthesis in the murine and rat gastrointestinal tract

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In the gastrointestinal tract, chronic alcohol consumption disturbs normal intestinal motor function leading to motility disorders.

Nitric oxide (NO) is the major mediator of inhibitory neurotransmission in the gut. NO is synthesized by three distinct isoform of NO synthase: neuronal (nNOS), endothelial (eNOS) and inducible (iNOS) NO synthase through distinct regulatory mechanisms. nNOS-immunoreactive nerve cells in the gastrointestinal tract have been localized to a subpopulation of neurons in the myenteric plexus.

The role of nNOS-derived NO in the physiological relaxations of murine gastrointestinal muscle has been demonstrated in pharmacological studies and in knockout animals. Inhibitors of nNOS have been shown to reduce nerve-mediated relaxations. However, the involvement and the regulation of NO synthesis under pathophysiological conditions are unclear.

We hypothesized the role of myenteric neuronal NO in the observed motility disturbances following chronic alcohol consumption. To test this, we have quantified the total number of myenteric neurons and nNOS-immunoreactive neurons in the mouse jejunum and in all segments of the rat intestine. We measured the release of NO in real time with a NO-reactive fluorescent dye and the effect of chronic alcohol consumption *in vitro* on nitrergic relaxations to electrical field stimulus (EFS) and exogenous NO in the mouse jejunum. Furthermore, NOS activity and protein content was determined in all segments of the rat intestine.

Mice received a gradually increasing concentration of ethanol in water with a final concentration of 20% for five weeks, controls received isocaloric sucrose solution or water. Rats received either 20% aqueous ethanol solution or water for 8 weeks. Using PGP9.5- and nNOS-immunostaining, the proportion of nNOS-immunopositive myenteric neurons was assessed. NO production was visualized and measured by confocal microscopy on tissue loaded with the fluorescent dye DAF-FM. The effect of ethanol treatment on nitrergic relaxations to EFS and exogenous NO of jejunal circular muscle strips was investigated *in vitro*. Small intestinal transit was measured *in vivo* after intragastric gavage of Evans blue. NOS activity in rat intestinal segments was measured by the conversion of [^{3}H]L-citrulline from [^{3}H]L-arginine and protein content was determined by Western blotting.

The percentage of nNOS-immunoreactive neurons decreased significantly after chronic alcohol consumption compared to controls, while the total number of neurons did not change in both mice and rat samples. DAF-FM fluorescence was significantly increased in neurons after chronic alcohol consumption and only partial colocalization with nNOS was observed. In jejunal circular muscle preparations, the nitrergic nerve-mediated relaxations to 1, 2 and 4 Hz EFS significantly decreased after ethanol treatment compared to controls, while relaxations to exogenous NO remained unchanged. Small intestinal transit was significantly delayed in mice after chronic alcohol consumption when compared to water control mice but significantly accelerated when compared to the sucrose control group.

Constitutive NOS activity significantly decreased after chronic ethanol treatment in the jejunum, ileum and colon but not in the duodenum. Inducible NOS activity levels were not significantly affected by ethanol. The nNOS protein content measured by Western blotting indicated a significant decrease in the colon after ethanol consumption, while in other intestinal segments change was not detectable.

Our results suggest an adaptive and region-dependent change in the synthesis of NO most particularly by the neuronal isoform of NOS in response to chronic alcohol consumption. The reduction of its activity and protein content as well as the reduction in nNOS-immunopositive cell number indicates a downregulation of the enzyme. The impairment of nNOS might partially account for the observed motility disturbances.

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DISSERTATION SUMMARY

Atilla, the novel GPI-anchored lamellocyte antigen, affects melanotic tumor formation in *Drosophila*

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Since the discovery of the homologous vertebrate signalling pathways, *Drosophila melanogaster* (fruit fly) serves as one of the major model organism in studies of innate immunity. The cellular arm of the *Drosophila* immune reaction includes the phagocytosis and the encapsulation reactions, the latter resembling the granuloma formation in vertebrates. During the encapsulation reaction the foreign bodies which are to large to be phagocytosed – for example eggs of parasites - will be separated and inactivated. To study the molecular events leading to encapsulation we started to analyze cell surface antigens of the major effector cell in this reaction, the lamellocyte. We identified the first surface antigen specific to these cells, dubbed Atilla, by combining an immunological and a reverse genetic approach. The HPLC analysis of the isolated 16 kDa protein identified a *Drosophila* gene. The analysis of cell specific expression of *atilla* gene proved lamellocyte-specificity in larval stages. The involvement of the molecule is suggested by its association with lipid rafts. Atilla antigen shows structural similarity to GPI-anchored receptor molecules in the family of Ly6/u-PAR, having a pivotal role in tumor invasion and metastasis. We established loss of function mutants for *atilla* gene, by P-element mobilization. Analysis of the homozygote viable mutants revealed a genetic interaction between a tumor suppressor mutation and the loss of function mutation in the *atilla* gene resulting in a drastically decreased tumorous phenotype.

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DISSERTATION SUMMARY

Effects of different galanin compounds and fragments on vasopressin and oxytocin secretion in rats

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Following the discovery of galanin (GAL) in the porcine intestine (Tatemoto et al. 1983), many data have been reported as concerns its physiological effects. These results suggest that the 29 amino acid-containing GAL plays a significant role as a peptide modulator in the regulation of the function of the hypothalamo-neurohypophyseal system. Aims: The effects of rat, porcine, human GAL and the 1-16 N-terminal and 16-30 C-terminal fragments of human GAL on vasopressin (VP) and oxytocin (OT) secretion were studied in rats. The question was investigated of whether the GAL receptor antagonist galantid (M15) was able to prevent the VP and OT level changes induced by GAL. Finally, the direct effects of GAL on VP and OT production were examined in isolated neurohypophyseal (NH) tissue cultures (an in vitro study).

Following intravenous (i.v.) or intracerebroventricular (i.c.v.) administration of GAL, the plasma VP and OT levels were determined by radioimmunoassay (RIA). Basal plasma VP and OT concentration elevation were induced by osmotic (2.5% NaCl solution) or non-osmotic (histamine - HA) stimuli, or lactation. To make the isolated NH tissue cultures, we used an enzymatic dissociation technique. The VP and OT contents of the supernatants of 14-day cultures were determined by RIA.

GAL administered i.v. (9.6 µg/kg) did not influence the basal or the stimulated VP and OT excretion. After the i.c.v. injection of GAL (0.32 µg/kg), the basal VP and OT levels did not change. The plasma VP and OT concentration enhancements induced by intraperitoneal NaCl administration, or lactation were prevented by prior treatment with i.c.v. GAL. The HA-induced elevations in plasma VP and OT levels were significantly moderated by i.c.v. GAL administration. During lactation, the higher OT level was decreased after GAL administration.

There was no essential difference in the VP and OT release effects of rat, porcine and human GALs. The two investigated human GAL fragments had different effects: the human GAL 1-16 fragment proved active as concerns VP and OT regulation, whereas the human GAL 16-30 fragment proved ineffective.

The GAL receptor antagonist M15, administered i.c.v. before the GAL injection, prevented all of the VP- and OT-responsive effects of GAL.

After the administration of increasing doses (10^{-13} – 10^{-6} M) of GAL, linear decreases were detected in the VP and OT contents of the supernatant medium in the isolated NH tissue culture.

The present results demonstrate the important role of GAL in the regulation of VP and OT secretion following different forms of stimulation: an osmotic response, HA administration or lactation. Our findings lead us to conclude that the 1-16 N-terminal GAL fragment contains the biologically active centre of the GAL molecule (Molnar et al. 2005). The results of experiments with isolated NH tissue cultures indicate that the GAL-ergic system can directly influence the hormone-producing activity of the pituicytes.

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DISSERTATION SUMMARY

Identification of "anchoring genes" in the promoter-upstream region of the *Drosophila Abd-B* domain

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The *Abd-B* gene determines the segmental identity of four different abdominal segments (from the 5th to the 8th) through the action of extensive segment-specific *cis*-regulatory regions (*iab-5, iab-6, iab-7, iab-8*). Although these regulators are located many kilobases away the promoter, physical contact occurs between them through the looping out of the intervening DNA sequences.

Genetic studies gave evidence that regulatory interactions between promoter and enhancer elements can be formed not only in *cis* but in *trans*, as well, between non-continuous DNA molecules. These *trans*-interactions depend on the physical proximity of homologous chromosomes. Taking advantage of this phenomenon, called "transvection", we have tried to identify genes that help to anchor distant enhancers to the *Abd-B* gene.

Our previous results (Sipos et al. 1998) strongly suggest the existence of a novel mechanism that tethers *cis*-regulatory regions to the promoter upstream region of the *Abd-B* gene of the bithorax-complex. The strength of *cis*-interaction is inversely proportional to the size of the deletion in the upstream region suggesting that this region consists of numerous discrete elements that cooperate in locking individual *cis*-regulators to the *Abd-B* gene.

The very sensitive relationship between the size of the sixth tergite of Drosophila males and the expression level of the *Abd-B* gene enables the identification of "anchoring genes" required for tethering of *iab-7* regulatory region to the promoter-upstream region. Most likely, mutation of "anchoring genes" would eliminate or weaken the *trans*-suppression of the *Fab-7* phenotype in *Fab-7/Abd-B* males, resembling to the phenotype observed in the presence of chromosomal rearrangements.

Screening for this phenotype gives also the opportunity to identify new genes involved in the pairing of homologous chromosomes, known or yet unknown repressors of the *Abd-B* homeotic gene, or mutants, which simply carry chromosomal rearrangements.

We performed a large-scale F1 mutageneric screen using EMS and ENU, as they more often induce point mutations than chromosomal rearrangements. From more than 90000 males we selected 500 modifiers. Due to the high degree of sterility only 63 of them carried reproducible phenotype. 22 of them were mapped to the 2nd chromosome and 41 on the 3rd chromosome.

To restrict the number of total hits we made complementation analysis. On the 2nd chromosome we isolated 2 single hits and one big complementation group (20 members). On the 3rd chromosome we found 3 different complementation groups and 25 single hits.

To filter out those hits that generated chromosomal rearrangements we performed specificity test by using pairing-sensitive transvection-systems characteristic for the 2nd (bw^V) and for the 3rd (Cbx^1Ubx^1) chromosome. Chromosomal rearrangements disturb the communication between the two homologs and therefore suppress the mutant phenotypes toward wild type. The bw^V phenotype was suppressed in 2 lines. Suppression of the Cbx^1Ubx^1 phenotype occurred in 8 cases. The Cbx^1Ubx^1 system also enabled the identification of new putative repressors belonging to the Polycomb-group.

The phenotype of complementation groups was "roughly mapped" between recessive markers on the mapping chromosomes. In further steps, deletion analysis and allelic complementation enabled the identification of mutated genes. We identified on the 2nd chromosome *grainyhead* (single hit) – a negative regulator of the *Abd-B* gene and *ebi* (big complementation group) – a previously unknown interactor of the *Abd-B* homeotic gene. The identification of the mutated genes on the 3rd chromosome is still under investigation. Using immunohistochemistry, we want to find out the regulatory hierarchy between the Abd-B and the mutated genes.

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DISSERTATION SUMMARY

Paleopathological investigations of the skeletal material of Szeged-Vár, Hungary

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Paleopatology is a special field of historical anthropology. Its main object is investigating of diseases, which past human populations suffered from. Paleopathology can be divided into several different categories depending on the aetiology of illnesses (Aufderheide and Rodríguez-Martín 1998).

One of the most important fields of paleopathology is the investigation of specific infectious diseases because they appeared as selective factors in past human population. We can only recognise those which can also produce osteological symptoms. Such infections are TB, leprosy and syphilis.

The aim of this study is to introduce some diseases observed on the skeletons from the cemetery of the gothic church built in the 14th century next to the castle of Szeged. The burial place was first used until 1543, then after the period of the Turkish occupation until 1713.

439 graves and several objects have been recovered till now, but the excavations are still going on. To collect paleopathological traits, macroscopic-morphologic and stereomicroscopic observation and X-ray analysis have been carried out. In two cases radiocarbon dating was also performed.

After determination of sex and age, we could establish that the sex ratio was 1:1, and the percentage of juveniles (under the age of 18) to adults was 41% to 59% in our material

Many of the skeletons showed different forms of paleopathological alterations, mostly minor developmental anomalies and joint diseases. Probably the most common pathological disorder was degenerative arthritis but there were other diseases related to joints like diffuse idiopathic skeletal hyperostosis (DISH) caused by abnormal function of the metabolic system (Aufderheide and Rodríguez-Martín 1998).

There were only few skeletal evidences of trauma; we could identify mostly healed fractures (on the ribs and upper limbs). In one case we also observed an unusual skull fracture, which might have been caused by a sword cut injury (Ősz et al. 2005).

We could find some diseases related to metabolic and haematological disorders. Such symptoms were porotic hyperostosis (cribra orbitalia and cribra cranii) sometimes caused probably by vitamin C deficiency and sometimes connected to other illnesses like syphilis.

We could notice traces of several non-specific infections like periostitis and endocranial pattern and also – in one case - osteomyelitis.

The remains of four individuals showed serious bone lesions related to the osteological symptoms of treponematosis and even acquired syphilis (Hajnal et al. 2004; Ősz et al. 2005). On the skull of two adult skeletons the different stages of caries sicca (Hackett 1976) could be detected. The postcranial skeletons were also affected; cortical thickening and periosteal new bone formation were observable as well.

Each remain could be dated to the late Middle Ages on the basis of the archeological finds and observations. In one case the radiocarbon dating suggested pre-columbian origin. At that time Szeged was a merchant city and a very important port of the southern part of Hungary.

This could explain the early appearance and the relatively high prevalence of syphilis at the site (Ősz et al. 2006).

The low frequency of fractures and of other serious illnesses suggested that the main population of the castle and its surroundings were rather citizens than soldiers and they must have been in good nutritional and social condition.

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DISSERTATION SUMMARY

The *Thiocapsa roseopersicina* genome project and the use of results in the hydrogenase research

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Hydrogenases are metalloenzymes catalyzing the oxidation of molecular hydrogen and the reverse reaction (Vignais et al. 2001).

Thiocapsa roseopersicina BBS is a Gram-negative, photosynthetic purple, sulphur bacterium. The bacterium contains is able to fix atmospheric N_2, a process accompanied by H_2 production.

Thiocapsa roseopersicina harbours at least three different types of NiFe hydrogenases: HynSL, HupSL, HoxEFUYH. It is an intriguing question why the cells need so many distinct hydrogenases (Kovacs et al. 2005).

The NiFe hydrogenases contains a complex metallocenter composed of a Ni and Fe atom. The assemly of this center requires concerted action of numerous so-called accessory enzymes. Moreover, the hydrogen metabolism is linked to other bioenergetic/metabolic processes like photosynthesis, sulfur- and nitrogen metabolism, respiration etc. For characterization of the various metabolic pathways related to the hydrogen metabolism the whole genome sequencing of *T. roseopersicina* was started.

First of all a shotgun library was constructed. More then 15 000 clones were sequenced from both sides and the sequences assembled into 2400 contigs after clearance. As a next step a cosmid library was constructed which can be used to assemble the existing sequences into larger contigs and to fill the gaps between them. The sequencing of more than 3000 cosmid clones are in progress. Although the genome sequencing has not finished yet some of the important genes were found in the shotgun library.

In few microbes it was shown that the membrane associated hydrogenases are transported into the periplasmatic space via twin-arginin-translocation (Tat) pathway. The Tat pathway has a typical signal sequence, a conserved (S/T)-R-R-x-F-L-K motif, which can be found at the beginning of the small subunit of the hydrogenases also. The genes coding for the components of the Tat pathway was identified in the genome.

In the case of the membrane bound Hyn hydrogenase in *T. roseopersicina*, the structural genes are arranged unusually, since there two additional open reading frames (namely *isp1* and *isp2*) are located between the genes coding the small and the large subunit.

The function of the Isp proteins were studied by mutagenesis. It was shown that the Isp1 and Isp2 are required for the activity of the Hyn hydrogenase *in vivo* although they do not have on the hydrogenase activity *in vitro*. It was concluded that the Isp proteins were involved in the transmembrane electron transport. The Isp1 protein is a hem-containing integrated membrane protein in which characteristic signal sequence could not be observed. Hence, it is assumed that it is transported into the membrane via the Ffh/FtsY (SRP) pathway. The genes encoding the FtsY were recognized in the genome

The role of the SRP pathway in the transport of Isp1 and the Tat proteins in the export of the HynSL are going to be clarified. This would be the first case when the components of a functional membrane associated enzyme complex would be targeted by distinct pathways.

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DISSERTATION SUMMARY

The different role of ADA2b proteins in genome-wide acetylation and specific gene activation

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In eukaryotes the genetic material is present in a compact chromatin structure consisting of DNA and histone proteins. This chromatin structure must be opened in the early transcription steps to enable the binding of transcription factors to the promoter region. This process is regulated by the histone acetyltransferases. One of these acetyltransferases is the GCN5 subunit of GANT histone acetyltransferase complexes. GCN5 is one of the most intensively studied histone acetyltransferase and it is the catalytic component of a number of protein complexes. Other components of the GCN5-containing complexes are adaptor proteins like ADA2 and ADA3.

Recently, our group has reported that the Drosophila genome contains two distinct genes encoding ADA2 homologs. Biochemical characterization of the two ADA2 proteins demonstrated that both of them interact with the HAT GCN5 and participate in transcription activation. On the other hand, ADA2a and ADA2b exhibit marked differences, e.g., they participate in distinct high-molecular-weight HAT-containing protein complexes, are localized to different chromosomal loci, and have at least partly different partners of interaction.

Here we demonstrated that the complexity of ADA proteins is even higher and the Drosophila Ada2b gene produces two types of Ada2b mRNAs. The two proteins translated from the mRNAs are localized in different parts of the cells. The ADA2b1 protein is presented in the cytoplasm while the ADA2b2 is presented in the nucleus.

I generated Ada2b mutations which permit studying the role of the two ADA2b proteins together and separately as well. Ada2b mutations caused lethality in the late-pupa stage and reduced H3 K14 and H3 K9 acetylation. Interestingly, only the ADA2b1 isoform is required for the general H3 K14 and K9 acetylation. The absence of ADA2b2 did not affect the level of these acetylations.

The Ada2b mutation affects TAF10 localization at some, but not all, specific bands on Drosophila polytene chromosomes. The introduction of an Ada2b transgene into Ada2b mutant animals restored a staining pattern of polytene chromosomes identical to that seen in wild-type animals, providing further evidence that the loss of TAF10 localization at these sites derived from ADA2b depletion. This suggests that in certain complexes the presence of ADA2b is required for the incorporation of TAF10.

The mutation of Ada2b caused dramatical changes in specific gene function. Comparison of the pigment contents of Ada2b heterozygotes and wild-type animals indicated that Ada2b mutation affected the pigment contents of the eyes of adult animals. In concert with this, the mRNA level of rosy, a gene encoding xanthene dehydrogenase, which is involved in the formation of red eye pigments, exhibited a significant reduction in Ada2b mutant animals compared to wild-type animals. The two isoforms of ADA2b differ in regulation of rosy, the presence of ADA2b2 is necessary for normal expression level of rosy gene. On the other hand the ADA2b1 and ADA2b2 act differentially in the regulation of mitotic specific genes. The Ada2b mutation abolishes the Map205 expression but the presence of ADA2b2 rescues the Map205 mRNA level, while the ADA2b1 does not.

The Dmp53 function is essential for radiation-induced apoptosis. DNA damage leads to Dmp53 activation, which causes apoptosis through the transcriptional activation of proapoptopic factors. For Ada2b mutants, the number of cells undergoing apoptosis was significantly lower than that in wild type animals. For the tested Ada2b, the number of acridine orange-stained cells was decreased significantly. Interestingly, high-dose X-ray irradiation, which resulted in a decrease in apoptosis in wing imaginal disks, induced the reaper message level similarly (a three- to fourfold increase compared to the non-irradiated controls) in wild-type and Ada2b larvae.

The mutation of Ada2b causes dramatical changes in specific gene activations. Approximately six percent of the known Drosophila genes showed decreased (368 genes) or increased expression (461 genes) in the absence of Ada2b. These results indicate that the ADA2b containing complexes coordinate the regulation of genome wide expression through specific genes.

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DISSERTATION SUMMARY

Isolation of new planar polarity mutants in *Drosophila melanogaster*

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Polarity is a fundamental atribute of living organisms and cells. One of the most common form is the apical-basal polarity. Many cells are, however also polarized within the plane of the tissue, and thus this type of polarity is called planar cell polarity (PCP) or tissue polarity. In *Drosophila* all adult cuticular structures are polarized within the plane whereas in vertebrates a similar polarity is seen in the arrangement of fish scales, bird feathers, hair of mammals, the orientation of cilia in the oviduct or the stereociliar bundles in the neurosenory epithelium of the inner ear (Fanto McNeill 2004).

Despite the grate progress that has been made in vertebrates during the past few years, Drosophila system remained the best studied example of PCP. PCP in flies is most evident in the wing, which is covered by uniformly polarized, distally pointing hairs, in the epidermis, where sensory bristles point to the posterior, and in the eye, where PCP results in a mirror symmetry arrangement of ommatidia. Polarization in these tissues is controlled by the PCP gene products, mutants of which gene impair planar organization. Some of the PCP gene which have been placed into the core group, appear to affect polarity in all of the tissues. The key members of the core group are frizzled (fz), strabismus (stbm)-Van Gogh (Vang), dishevelled (dsh), flamingo (fmi), also known as starry night (stan), diego (dgo) and prickle-spiny legs (pk-sple).

In addition to the core genes, several other genes, like fuzzy, inturned, fritz, multiple wing hair, roulette, nemo, RhoA, mishapen and jun have been classified as secondary polarity genes becouse their function is required in only a subset of tissues (Adler and Lee 2001). These genes are thought to function as effectors of the core genes.

Taken together, a very simple model of *Drosophila* PCP establishment can be drawn: the products of the core genes, in response to an as yet not precisely identified polarity signal, build up a signalling center that controls polarity through tissue specific effectors. While a lot has been learned about the core genes, many important questions remain. How do they interact at the molecular level? How is this pathway connected to others? What are the effector genes and how can they control coordinated physical changes in cell behaviour?

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In order to contribute to the answering of above mentioned questions by isolating new tissue polarity genes, we initiated a large scale mutagenesis screen for the left (2L) and right arm (2R) of the second chromosome and the right (3R) arm of the third chromosome of *Drosophila melanogaster*. We used the FRT/Flp mosaic system (Xu and Rubin 1993) which is employed for the first time to identify polarity mutants. To encrease the sensitivity of the screen, we performed an F_2 screen which offers the advantage of recognizing phenotypes with low penetrance and eliminates the sterility problems of F_1 screens. As a mutagene we used EMS in 30 mM concentration and ENU in 1,6 mM concentration.

After the mutagenesis of the 2R, 2L and 3R and screening of about 22,800 crosses, we found 58 strong and many weeker PCP mutants. These mutants showed a random orientation of bristles on notum and hairs on wings, multiple wing hairs, misrotated and symmertrical ommatidia and chirality flips in eyes.

In order to map our mutants and to test whether they are carrying new genes we initiated complementation analysis. The complementation analysis has shown that on the 2L we isolated 18 fmi, 1 stbm, 1 pk and 3 new alleles, on the 2L 1 ds, 4 frtz and 3 new alleles, and on the 3R 27 new alleles. The crosscomplementation of these alleles revealed that the new alleles on the 2L and 2R are unique, while on the 3R we could distinguish one complementation group with 5, one with 2 members and the others were unique. We roughly mapped our mutants based on lethality with deficiencies and the most interesting mutants were also mapped by recombination to determine whether the mutation and lethality are correlated. In order to determine if our mutants can be members of the PCP pathways, we examined their genetical interaction in eye with the two well characterized PCP genes dsh and fz. We have found that most of our mutant enhances or suppresses these genes, so they can be fitted in PCP pathway at different levels.

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DISSERTATION SUMMARY

Molecular and genetic analysis of the Rpt1 subunit of *Drosophila* 26S proteasome

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The eukaryotic 26S proteasome defines the final end of the lifetime of many proteins by recognizing the multiubiquitin tag attached to the appropriate protein and thereafter executing its destruction through its deubiquitylation, unfolding, translocation to the peptidase sites and lastly, cleavage of the protein backbone after every 8-10 amino acids. Within the proteasome, the activity of the core protease particle is regulated by one or two regulatory complexes (RC). The RC-s are responsible for the above mentioned first steps of the destruction. The RC is comprised of two well-defined protein subcomplexes, the lid and the base. The base contains six AAA-type ATPases, similar to each other in sequence, which are assumed to function as reverse chaperones, unfolding the substrate and translocating it through the gated orifice of the core peptidase right to the protease sites.

Despite their similarities, several unique features were discovered concerning these ATPases, even though their direct functional pathways and their interactions with other proteins are still unknown. One of them, p48B/Rpt1 has remained largely uncharacterized until now in *Drosophila*. The ease of mutagenesis with P-elements and subsequent mutation detection in this organism aided us in characterizing three P-element insertions in the 5'-untranslated region (UTR) of the Rpt1 gene. These insertions proved to be recessive lethals, two of them dying in second larval and one in pupal stage as homozygotes. One larval lethal allele was modified by transposase-induced P-element excision and the resulting white-eyed flies were examined further. These mutants could be sorted into two lethality groups, namely second larval and pupal stage lethals. After sequencing their appropriate genomic regions, pupal lethality was found to be associated with short (30-35 bp) while the larval lethality with long (more than 600 bp) remaining P-element sequences at the original insertion site, which were formed due to imprecise P element excision.

Several tests were performed to verify the relation between the mutation in the gene and the lethality stage, considering the possibility that a second-site mutation could be responsible for the phenotype. The effect of the mutation on the subunit expression and modification was investigated and we discovered profound changes both at mRNA and protein levels in case of Rpt1 mutants compared to the wild-type. RT-PCR revealed a decrease in full-length Rpt1 mRNA expression in one pupal lethal mutant as well as immunoblot against the Rpt1 protein showed reduced subunit expression in the mutant. At molecular levels we found several ubiquitin-proteasome system-related abnormalities in the mutant animals. Native gel electrophoresis demonstrated that the two well-defined forms of 26S proteasome (the singly-capped form: RC-20S proteasome, or the doubly- capped form: RC-20S proteasome-RC) disappear in pupal lethal mutants, and the amount of free 20S proteasome increases. The mass of ubiquitylated proteins is also increased in the mutants in comparison with the wild-type. We found that the ratio of the two isoforms of Rad23, which is a nucleotide excision repair factor and, in addition, a multiubiquitylated substrate shuttling protein associated to the proteasome, is also affected: the higher molecular weight form termed A, which is 10-fold less abundant in the wild type than the B one, is almost as abundant as the B form in the mutant pupae. The expression of proteasome subunits other than Rpt1 seems to be only slightly impaired. To sum up our results we analyzed several mutant alleles of one proteasomal ATPase gene and our findings indicate that this subunit is essential for the proper functioning of the 26S proteasome and for the normal development of the fruitfly *Drosophila*, furthermore this investigation provided us interesting insights into the function and regulation of the proteasome regulatory complex.

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DISSERTATION SUMMARY

Salicylic acid improves the acclimation of *Lycopersicon esculentum* Mill. L. to high salinity by approximating its salt stress response to that of the wild species *L. pennellii*

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Salicylic acid (SA), a plant phenolic is now considered as a hormone-like endogenous regulator, and its role in the defence mechanisms against biotic and abiotic stressors has been well documented (Yalpani et al. 1994; Szalai et al. 2000).

In the present work acclimation to salinity stress of tomato plants, which had previously grown for a long time in a relatively low concentration of SA, was investigated. We tried to reveal those biochemical and physiological effects of SA pre-treatment which led to improved fitness of plants exposed to salt stress. In this work we demonstrate that SA pre-treatment induced a halophytic character in *L. esculentum* cv. Rio Fuego, and as a result, accumulation of sodium as inorganic osmolyte in leaf tissues without any serious symptoms of salt stress.

Tomato plants were grown hydroponically. Plants pretreated with 10^{-7}-10^{-4} M SA were exposed to 100 mM NaCl for a week. Ion contents of tissues, relative water content, water potential and osmotic potential of the youngest expanded leaves were determined after SA pretreatment and in plants exposed to salinity. SA treatment decreased the $K^+/^{86}Rb^+$ uptake and contents, and water potential of plants but during the next three-week an osmotic adaptation occurred. Na^+ ions accumulated in the leaf tissues of treated plants exposed to salinity and functioned as inorganic osmolytes. As a result of water potential decrease, the concentration of abscisic acid (ABA), and simultaneously, the activity of abscisic aldehyde oxidase, catalyzing the last step of ABA synthesis, increased in the roots of SA-pretreated plants. This enabled plants to activate the ABA signal transduction pathway and gene expression before salt stress. ABA-regulated genes, such as LEA-s (late embryogenesis abundant proteins), RD29A, DREB2A are clearly involved in the acclimation to salt stress (Ma et al. 2006). In the presence of SA, the leaves accumulated more compatible osmolytes e.g. soluble sugars, glucose and fructose, a sugar alcohol, sorbitol and proline. SA-pretreatment improved the photosynthetic efficiency of plants. By chlorophyll fluorescence measurements, it can be concluded that the quantum efficiency of PS2 open centers was not different from that of the control in the treated leaves in dark adapted state and it increased in light adapted state.

10^{-4} M SA pretreatment induced antioxidative defence mechanisms; first of all it enhanced the activity of ascorbate and guaiacol peroxidase activity in the roots. Pretreated plants maintained high levels of non enzymatic antioxidants, such as carotenoids and a polyamine, putrescine in the shoots under salt stress.

An enhanced acclimation to 100 mM NaCl induced salt stress could be observed at a 10^{-4} M SA pre-treatment. In these plants greater accumulation of putrescine, spermidine and spermine occurred before salt exposure, which decreased the oxidative damage and protected membranes under salinity.

Protoplast cultures were made to detect the effect of SA to the cell viability. By Evans blue staining it was investigated that the effect of SA could be prevented by application of ABA, proline and putrescine. The effectiveness of SA in improving the salt stress response of *L. esculentum* similar to the acclimation mechanisms observed in the Na^+ includer, salt tolerant wild species, *L. pennellii*.

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DISSERTATION SUMMARY

Role of the posttranslational modification in the DNA repair

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DNA damage is a relatively common event in the life of a cell and may lead to mutation, cancer, and cellular or organismic death. Damages to DNA induce several cellular responses that enable the cell either to eliminate or cope with the damage or to activate programmed cell death process.

Postreplication repair is the mechanisms that assure the replication of the damaged DNA. One of the postreplication repair pathways is the translesion synthesis. This process comes into the play, when the replicative DNA polymerase can not incorporate nucleotide opposite the damaged bases and the moving of the replication fork is blocked. In this case switching of the replicative polymerase and translesion polymerases occurs. The TLS polymerases can incorporate nucleotides opposite the damaged bases. This process can be error–free or error–prone. The error-prone synthesis generates mutation, in contrast to the error-free pathway.

The major regulator in the polymerase switching is the Proliferating cell nuclear antigen (PCNA). PCNA has been described as a processivity factor of DNA polymerases, and it has an essential role in the DNA replication machinery. Recent studies suggest that PCNA plays an important role in polymerase switching, and PCNA could determine which polymerase gain access to the replication sites. The major unanswered question is: How could PCNA regulate the polymerase switching? The recent experiments have suggested the importance of the secondary modifications of the PCNA as well as the polymerases.

PCNA is exquisitely modulated by covalent post-translational modifications involving ubiquitin and the ubiquitin-related protein SUMO. PCNA is a target for SUMO modification, mono-ubiquitylation, and K63-linked multi-ubiquitylation. In contrast to K48-linked multi-ubiquitylation, mono-ubiquitylation and modification by K63-linked chains do not generally promote proteasomal degradation, but rather they seem to alter the function of the substrate or to mediate protein–protein interactions. Two target sites have been identified in PCNA for modification: K164, a conserved modification site that is both modified by SUMO and ubiquitin, and K127, a yeast-specific site that seems to be exclusively modified by SUMO.

Several studies have analyzed the function of the ubiquitylation of the PCNA, but the sumoylation events have not been characterised yet. The goal of our study is to understand better the switching mechanism of polymerases and shed light on role the sumoylation of the PCNA. We have reconstituted the sumoylation of PCNA in vitro, and we have started to characterize this modification. Now, we would like to purify sumoylated PCNA and closely study the major question: how PCNA can regulate the polymerase switching mechanism during the postreplication repair.

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DISSERTATION SUMMARY

En route to the first lamellocyte-specific driver

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The immune system of *Drosophila melanogaster* (fruit fly) is an excellent model for the vertebrate innate immunity because of their homologue signalling pathways. The cellular immune response (phagocytosis, encapsulation and melanisation) is mediated via hemocytes. We are interested in the differentiation of hemocytes, namely in the formation of lamellocytes. To identify activation pathways acting in lamellocyte formation previously we have carried out a UAS/Gal4 screen with UAS-transgenes which are members of known signalling pathways (Zettervall et al. 2004). The UAS-constructs were driven by the *hemese*-Gal4 driver, that uses the promoter/enhancer region of *hemese*, a larval panhemocyte-specific gene (Kurucz et al. 2003). This screen demonstrated that activation of Jun kinase, JAK/STAT, Toll and Wnt signalling pathways in hemocytes lead to lamellocyte formation equally. To dissect this complex picture, we decided to construct a lamellocyte specific Gal4-driver which would enable us to examine the activation pathways regulating lamellocyte formation. To generate this construct, we used the genetic region of *atilla*, a lamellocyte-specific gene, which was identified in our workgroup (Laurinyecz et al. in manuscript). We cloned the upstream 2 and 4 kilobase 5'UTR containing regions of the *atilla* gene. The transgenic Gal4 constructs were inserted into *Drosophila* embryos, and tested in larvae. One transgenic strain arisen from the 2 kilobase construct showed Gal4 expression in the ejaculatory duct in adult males. Normally this tissue does not express Atilla. Although it was not the required lamellocyte-specific expression, it demonstrated that our transgene can act as an enhancer trap, and it carries the promoter sequence of the *atilla* gene. The 2 kilobase Gal4 construct was designed with additional restrictional cleavage sites to use them in the future.

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DISSERTATION SUMMARY

Purification and examination of Hox hydrogenase in *Thiocapsa roseopersicina*

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Hydrogenases catalyze the enzymatic redox reaction $H_2 \leftrightarrow 2H^+ + 2e^-$. The purple sulfur phototrophic bacteria *Thiocapsa roseopersicina* contains several hydrogenases. HynSL is the most stabile, membrane bound, bidirectional hydrogenase. HupSL is a membrane bound H_2 uptake hydrogenase responsible for H_2 consumption produced by nitrogenase enzyme complex. The heteropentameric bidirectional HoxEFUHY hydrogenase, situated in the cytoplasm and consists of a hydrogenase part (HoxHY) and a diaphorase part (HoxEFU) which catalyzes the reduction of NAD^+.

The HoxEFUHY in *Thiocapsa roseopersicina* is a NiFe hydrogenase. These enzymes consist of a large subunit containing the catalytic center with Ni-Fe atoms, coordinated by cysteines and unique CO and CN ligands and a small subunit, responsible for e^- transport, containing Fe-S clusters. The maturation of the enzymes is influenced by several proteins, namely: HypA and HypB are needed for Ni and Fe incorporation, HypC is a chaperon, HypD and HypE is necessary for the transportation of Ni, Fe atoms, and HypF involved in the formation of CO and CN ligands. HoxW is a protease responsible for a C-terminal cleavage of the large subunit.

The genes encoding the HoxEFUHY are well known (Rákhely et al. 2004), but the enzyme itself needs to be investigated. The aim of my project was the purification and the characterization of the enzyme complex. In the first attempt the classical chromatographycal methods were used, and optimized. The chosen methods were cheramic hydroxiapatite (CHT), ion exchange (IEX), hydrophobic (HIC), and gel filtration (GF) chromatography. Purification from the soluble protein fraction, a four-step chromatography protocol (CHT→IEX→GF→HIC) was developed. The final fraction showed H_2 evolving activity, but Hox subunits could not be detected.

The enzyme was stable and easily stored after CHT but after IEX and HIC stability decreased dramatically, and activity dropped considerably. Addition of different stabilizing agents (glycerol, NaCl, detergents, reducing agents) or hypothetical cofactors (FMN, NAD, NADH) to the purified enzyme was not effective. Storage tests with the purified enzyme under special conditions - anaerobically, or in H_2 atmosphere - had no positive results.

The next approach was the affinity purification of the Hox hydrogenase using flag strepII tag fused to one of the subunits. Fusing the tag to the HoxH N-terminal end cripples the activity of the enzyme, while the C-terminal end of the large subunit processed by the HoxW. HoxE tagged construct was another alternative attempt, because HoxE is not important for in vitro activity and in vivo activity of the HoxE mutant could be reconstituted by a plasmid containing Flag-StrepII tagged HoxE. Affinity purification of the C-terminal tagged HoxE Thiocapsa strain resulted in the identification of the five Hox subunits by MALDI TOF MS. Again, most of activity disappeared during the purification procedure. Gel electrophoresis experiments showed that bands of different subunits were detected only in the fresh samples. Now there is another - HoxY C-terminal tagged - construct under construction.

It is still unclear what is needed to keep the active state of the enzyme during purification. In the strongest homologue Hox - among Cyanobacterias - similar problems have been reported. Active enzymes are purified only from Ralstonia eutropha and Rhodococcus opacus, but they are distant relatives and their Hox enzymes have different subunit structure. The stability of Hox must be improved, because pure and active Hox protein is needed for studies on substrates, cofactors, catalytic center and function of subunits.

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DISSERTATION SUMMARY

Combined effects of nitric oxide and cyanide on the photosynthetic electron transport of intact leaves

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Plants not only respond to ambient levels of nitric oxide (NO), but also generate NO themselves (Neill et al. 2003). In the past years, this gaseous free radical has been implicated as an important signaling molecule in numerous physiological processes (Río et al. 2004). Still, the role of NO in photosynthesis is poorly understood, which is well indicated by the modest number of in vivo and in vitro experiments in this area with mixed results (Takahashi and Yamasaki 2002; Yang et al. 2004). In our study, we aimed to clarify the potential effects of various NO donor molecules on the photosynthetic electron transport in intact leaves by means of quenching analysis of chlorophyll a fluorescence.

The youngest fully expanded leaves of 2-week-old Pisum sativum L. cv Rajnai Törpe plants were excised and the petioles were submerged in Petri dishes containing distilled water, NO donor molecules and scavenger chemicals with various concentrations. Chlorophyll fluorescence of PS II of pea leaves was measured with a PAM fluorometer (PAM-2000, Heinz Walz GmbH, Effeltrich, Germany). Q_A^- reoxidation kinetics was followed by a double-modulation fluorometer (Photon Systems Instruments, Brno, Czech Republic). Xanthophyll cycle pigments were determined by HPLC.

Q_A^- reoxidation kinetics in pea leaf discs were measured in the presence of the NO donor, sodium nitroprusside (SNP) in order to see whether NO can replace bound bicarbonate at different binding sites, e.g. the non-heme iron complex, in PSII as measured earlier (Petrouleas and Diner 1990). SNP slows down the electron transfer between the primary and secondary quinone electron acceptors in vivo in a concentration dependent manner, which indicates that NO displaces the bicarbonate from the acceptor side non-heme iron. Results in the presence of DCMU, which prevents forward electron transport, indicate that NO inhibits charge recombination reactions of Q_A^- with the S_2 state of the water-oxidising complex, as well as interacting with the tyrosine radical Y_Dÿ in PSII. Applying scavengers haemoglobin and cPTIO resulted in a complete or partial elimination of these changes, respectively, which indicates a distinct role for NO as well as cyanide in the process.

The joint effect of cyanide and NO – both released by SNP – on photosynthetic processes was observed in the complex chlorophyll a slow fluorescence kinetics, and experiments in the presence of the NO-specific scavenger cPTIO allowed clear distinction between effects induced by cyanide or NO. The SNP-induced moderate decrease in F_v/F_m, an indicator of light harvesting capacity, implies a structural alteration of the light harvesting complex of PS II, and measurements with cPTIO hold cyanide responsible for this concentration dependent decline. Increasing amounts of SNP cause a more dramatic change in quenching parameters qP and NPQ, which provide information about the ratio of open reaction centres in PS II (ready for electron transport) and the ratio of absorbed energy dissipated as heat, respectively. Consistent with results from Q_A^- reoxidation kinetics, dropping qP values indicate a slowing rate of electron transport – mediated chiefly by NO. Instead of a resulting NPQ (ÄpH) decrease, the Äp H is increased due to cyanide which inhibits the Calvin cycle, thus proton loss from the lumen. Other measurements with nitrite and nitrate, and another specific NO donor (GSNO) confirm a specific – cyanide-independent – NO effect.

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References

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